

Greens capture Europe's imagination

Last week's election of members of the European Parliament was more interesting than anyone expected. But will those with lessons to learn eventually take them to heart?

SIMULTANEOUS elections covering 12 countries are bound to be a nightmare for psephologists. Inevitably, some countries (Britain, for example) move to the left of the political spectrum and others (France this time) in the opposite direction, but in ways that do not allow of generalization. It has been like that since the European Parliament was created. Voting patterns in different countries seem to be determined by people's likes and dislikes of their own governments' domestic policies rather than for (or against) particular European policies. But that could now be changing. Both in Britain and in Europe, for example, parties labelled in one way or another as "green" surprised even themselves by the vigour of their showing. (Despite winning 14 per cent of the European vote in Britain, British greens will have not seats at the European Parliament in Strasbourg, for Britain does not have proportional representation.) Is that phenomenon a sign that Europeans now believe that the European Economic Community has a function in civilizing (in some sense not clearly defined) the more primitive inclinations of their own national governments? Or are there other explanations?

The first thing to say is that the greens did best where they were, before last week, insignificant. In West Germany, on the other hand, where the Green party has become familiar to the voters in national and regional elections, there was no noticeable improvement. No doubt the procedural wrangles with which the West German greens have maddened West German politicians of other persuasions have also frightened many of the voters. But it would be mistaken of orthodox politicians to argue that this shows that there is a natural limit to the strength of the unorthodox parties, somewhere between 10 and 20 per cent. For as long as Europeans have to elect politicians to two kinds of parliaments — national and European — there will obviously be a temptation to vote for a go-getting party to make a national government and then for a green European party to keep it in check. But, in the limit, that means that there is no obvious limit to the size of the green minority. The minority could even, one day, be a majority.

But who are the greens? And why did they do so well? Orthodox political cynicism may be sustained by the observation that the greens have done best where their policies are least well understood. That, for example, applies in Britain, where the green party that did so well

last week has been less concerned with the permissible amount of lead in petrol or the ins-and-outs of water-borne nitrates as a cause of stomach cancer as with the need to reconstruct society so as to make it immune from the troubles that plague the modern world, which is most simply achieved by retreating to a simpler past. The message manages to span the political spectrum by remarking, for example, that there were no "multinationals" (the opposite of a euphemism for a company that exports a significant proportion of what it makes or does) in those more innocent times.

That makes it more difficult to understand how the greens did so well. But there are several partial explanations that can easily add up to more than a whole. In West Germany, where the only important shifts last week were to the extreme right and to the Free Democrats (Chancellor Kohl's minority partners in his coalition government), two recent happenings have influenced the election in an important way: first, the visit of Mr Mikhail Gorbachev confirmed Free Democrats in their allegiance to Herr H. D. Genscher, foreign minister in the coalition government and the one who took the initiative in pressing for a series of negotiations to limit the numbers of short-range missiles in Europe. But the extreme right has also been encouraged by Gorbachev's talk of a "common European home": push for unity (with East Germany) and it will happen, they are saying.

The explanation is simpler in Britain, chiefly because Britain has become an over-simple place. Only a few years ago it seemed that the centre parties with a natural claim on the European vote would breed the politicians of the future. In the event, by quarrelling with each other, they have rubbed themselves. Britain's outstanding politician, the prime minister Mrs Margaret Thatcher, has meanwhile fallen out with Europe. For a reason that nobody properly understands, she has it in her head that Europe is peopled by syndicalists anxious to do down her and like-minded compatriots. Her speech at Bruges last year, full of cogent particular complaints that European conformity is troublesome, nevertheless misjudged the opinions of those who have in the past elected her to office. Unsurprisingly, for instance, British voters would quite enjoy the prospect that customs posts at frontiers might be abolished. No doubt the government party's managers, with their record of adroitness in these matters, will see to it that things are not in future so badly mismanaged.



Meanwhile, two substantial issues remain to trouble Western Europe, the first of which is the reputation for being green that it appears by accident to be winning. There is nothing wrong, of course, with an ambition to be free from knowable environmental hazards, infectious diseases for example. Not is it reprehensible that people should wish that their lifespans should not be put at risk by novel sources of contamination. But there is a danger that the structure of the European Community, by giving extra brownie points to those who push for self-restraint, without consideration of the cost, will undermine the agreed and serious part of its ambition, achieving the benefits of a genuinely common market. Those elected last week to Strasbourg will be even more inclined than their predecessors to vote against the use of anabolic steroids in commercial animal husbandry, believing that to be the green course. Who will be there to count the cost?

The second issue is political or, rather, constitutional. What this Euro-election has shown is that European voters are still hopelessly divided on their long-term objectives. Neither the British Labour party (which did well) nor the right-wing parties in West Germany (similarly) are federalists. They will turn up in Strasbourg with very different kinds of fish to fry. The chances are that the next European election will be a little more meaningful, but not much. Yet there are substantial European issues to discuss, from short-range missiles to the organization of higher education. Where and to whom Europeans will in future pay taxes should provide grist enough for any politician's mill. Those last week elected (or dis-elected) to Strasbourg might spend some time in the years ahead turning questions like those into the agenda items for a genuinely European election. □

A brave venture

Readers should not fail to remark the importance of the first of the letters in this week's correspondence section.

ONLY occasionally does it happen that ambitious projects for international collaboration make good sense practically as well as in the head, but there can be few who will not grasp the importance of the project described by Professor John Bahcall and his associates on page 574 of this issue. Their plan is quite simple; to found with public funds an international institute of astrophysics that will assume responsibility for future capital-intensive exploration of the Universe beyond the Earth. All of those concerned are experienced, often painfully so, at the complicated art of successful international collaboration in fields cognate with that to which their present hopes aspire. What they are saying to their colleagues and their governments is that there are simpler and more effective ways of helping astrophysicists work together than the complicated bilateral and even multilateral arrangements which are now in fashion. Why should there be a treaty, or

diplomatic negotiations, when practitioners know immediately what can and should be done, but need only the funds with which to do it and the freedom to go ahead?

The opportunities are immense. Why not, after Hubble, put a 10-metre telescope in geosynchronous orbit? Why not build a radio interferometer with a baseline so great that it is for practical purposes infinite? Why not make a catalogue of what remains a largely uncharted Universe that will, by approaching completeness, allow the substrates of common observations to assume their proper significance? Why not set out, in a common enterprise, to discover the machinery that keeps our larger environment in being? There is no counter-call. Indeed, this is not a field overburdened with people — compared with condensed-matter physics, for example, there are only handfuls of them. But there is no other field in which a few scraps of discovery can capture the general imagination as vividly. Plainly, the only reason that we do not already have an international institute of astrophysics is that nobody has thought of it before.

Naturally, there are difficulties. Why not, some will say, high-energy physics instead? There is a good case for that as well. But sadly, the major players in this field seem to have committed themselves to the next generation — or half-generation — of particle accelerators. Who, at this late stage, would interview the governor of Texas to tell him that the Superconducting Super-Collider is to be subsumed in an international enterprise? It would of course make sense that the participants in high-energy physics — Europe, Japan, the Soviet Union and the United States — should promptly sign an agreement with each other that they will not build another super-accelerator without consulting their co-signatories about the prospects of collaboration. But that agreement would bring fruit only a decade from now. Astrophysics could be a going concern by then, and could have shown how to make collaboration on the grand scale function.

There will also be smaller worries. There will be governments who fear that their brightest and best will be seduced away by the attractions of an institute such as that proposed. Really, and if so, so what? There is hardly a field of science in which people are so mobile. There will be complaints about the cost which could be turned in 10 minutes by an accountant skilled enough to add up present costs and spell out the economics — or, better — extra opportunities that would flow from collaboration. But if governments prefer to cogitate and rehearse their doubts, they should reflect on the character of the signatories of the letter on page 574. Each of them is not only a practitioner of distinction in the field, but is or has been a public servant of an important kind — one of those in whom independence is valued above mere administration. Giacconi has been one of the most telling critics of his ultimate employer's manned space station, Sagdeev is (or should be) cherished by his employers for having done more than most of his compatriots to make Soviet science modern, both by example and exhortation. Who will dare say such people nay? □

Double blow for cold nuclear fusion

- Harwell investigation stopped
- Los Alamos collaboration fails

Washington

Two negative announcements about cold fusion last week may prove particularly troublesome to Stanley Pons and Martin Fleischman, the researchers who set off the controversial debate with their claim on 23 February that a palladium electrode immersed in heavy water will produce large quantities of excess heat.

Dr David Williams, leader of a large team at the UK Atomic Energy Authority's Harwell laboratory in Britain which has been examining cold fusion since mid-February, and which had received help from Fleischmann in setting up its electrolytic cells, announced that the team had finally abandoned its experiments, having found no signs at all of anything unusual.

In the United States, officials at Los Alamos National Laboratory (LANL) let it be known that efforts to collaborate with Pons and the University of Utah had, after much negotiation, come to nothing. The idea that LANL scientists should examine working cold fusion cells at the University of Utah was brought up by Pons at last month's congressional hearing on cold fusion in response to questions about the difficulty of reproducing the claimed results and the importance of verifying them.

But at a press conference following the special cold fusion session at the Los Angeles meeting of the Electrochemical Society (*Nature* 339, 84; 1989), Pons said that the collaboration was "subject to the signing of agreements", and a spokesman for LANL said that lawyers from the University of Utah were being very careful not to lose their grip on patent and intellectual property rights.

By the time of the Santa Fe cold fusion meeting (*Nature* 339, 325; 1989), James Brophy, vice-president for research at the University of Utah, said that legal problems had been overcome and that the collaboration could proceed once a timetable had been arranged with Pons. But then, according to Howard Menlove of LANL, the laboratory had indicated to Pons its readiness to proceed, but received no indication of when work might begin.

After some weeks, an official announcement was made last week by LANL because it was felt that constant talk of a collaboration without there being scientific activity behind it could be misleading to observers of the cold fusion scene. Menlove says that LANL is still ready to participate if Pons wishes it.

The decision by scientists at Harwell to end their investigation of cold fusion was

eventually made, after almost three months, because the laboratory could not continue to devote man-hours, money and material to the project. Ten scientists, led by Williams, had spent three months and £320,000 on the studies, which began in the middle of February, even before the now famous 23 March press conference at the University of Utah, when the cold fusion claim was made public.

Fleischmann, of the University of Southampton, is also a consultant to Harwell, and had consulted his colleagues there originally to ask for their assistance in looking for a flux of neutrons from an electrolytic cell. An experiment was set up with Fleischmann's help.

After 23 March, the Harwell effort grew, and Williams and his colleagues obtained from Johnson Matthey, suppliers of palladium to Pons and Fleischmann, samples of every kind of palladium the company could provide, as well as some items made to order.

Williams therefore feels that the Harwell experiments have covered every conceivable aspect of palladium metallurgy, a murky subject which has frequently been invoked as an explanation for the purported success of a few experiments amidst the failure of most. But the Harwell team has not been allowed access to any of the electrodes from Pons and Fleischmann's working cells, which are now apparently back with Johnson Matthey.

Fleischmann last visited Harwell shortly after Easter, but since then had stayed away in order that the experiments should be seen as entirely independent of the original cold fusion claims.

The Harwell group also tried titanium electrodes, as well as some heavy-electron alloys and a number of well studied hydrogen-storage compounds, all with negative results. The sensitivity of their neutron detectors was even enough to contradict the claims of low-level neutron emission which have been made by Steven Jones and his colleagues at Brigham Young University.

Although no new science has come out of the Harwell investigation, Williams says that the interdisciplinary collaboration was nevertheless rewarding. But he has formed "trenchant opinions" about the dampening effects of lawyers on scientific investigations, and worries also that this public failure of a "crazy idea" may make scientists generally more reluctant to discuss the crazy ideas which are an essential part of progress. **David Lindley**

Titan boosts space plan

Washington

THE successful launch of a Titan IV rocket by the US Air Force on 13 June, coinciding with the release by the National Aeronautics and Space Administration (NASA) of a new timetable for shuttle and other launches, marks the end of the period in which the US space programme has been rethought and reorganized in the aftermath of the Challenger accident. The availability to NASA and the Department of Defense of a range of unmanned rockets as well as the shuttle restores much of the flexibility lost when the shuttle was fixed on as the sole US launch vehicle.

The Titan IV, a 'stretched' version of earlier rockets in the series, is seen by the US military community, which had always been critical of dependence on the shuttle, as the workhorse of its launch fleet. The identity of the payload launched last week is classified, but has been widely reported to be an early-warning satellite, destined for geostationary orbit and capable of detecting the plumes of enemy missiles.

Also last week, NASA issued a revised manifest for shuttle and expendable launch-vehicle flights, and the Titan IV is included for the first time. It will be used to send off the comet rendezvous asteroid flyby mission (CRAF), which had not previously been allocated a firm launch slot. Although NASA made the decision to put CRAF on a Titan IV instead of the shuttle because it was confident of the new rocket's imminent availability, the issuing of the manifest at the same time as the Titan's maiden flight was coincidental; the US Air Force had made no prior announcement of the launch.

The Hubble Space Telescope (HST) loses a few months, and is now scheduled for launch in March 1990 instead of December this year. The earlier shuttle flight has been given over to a mission to recover the long duration exposure facility (LDEF), a satellite carrying 57 experiments which was put into orbit by the shuttle in 1984. If not recovered soon, LDEF will re-enter the atmosphere and burn up.

The HST has suffered incessant and frustrating rescheduling because, unlike planetary missions such as Magellan (successfully sent off to Venus last month) or CRAF, it does not need to be put into orbit at any special time. A novel feature of NASA's latest manifest, intended to ameliorate this problem, is the designation of six shuttle flight opportunities, starting in 1992, with no reserved payloads. This extra flexibility in planning should mean that isolated launch difficulties should not require wholesale revision of the manifest. **David Lindley**

Congress asks "why Japan?"

Washington

MASSACHUSETTS Institute of Technology's Industrial Liaison Program found itself at the centre of a storm last week as a Congressional subcommittee probed the way the programme provides advance access for foreign companies to research programmes paid for by US federal funds.

About a half of the members of the liaison programme are foreign and, for a yearly fee of just \$10,000–50,000, MIT programme officers help give easy access to \$300 million a year of research, most of it paid for by the government.

The hearing by the Government Operations subcommittee on Human Resources and Intergovernmental Relations examined many examples of the conflicts of interest that have "followed private industry funds into the university" as subcommittee chairman Ted Weiss (Democrat, New York) put it. But in an often fractious hearing the subcommittee's strongest censure was reserved for MIT's Industrial Liaison Programme (ILP).

According to David Noble, a professor of history at Drexel University, MIT has portrayed the programme as a model for the transfer of the fruits of government-funded research from universities to US industry. But, he says, MIT has failed to say that a quarter of the members are

Japanese and that it maintains an "overseas sales office in downtown Tokyo".

Each company joining the programme is given a liaison officer who will alert it to programmes, patents and research of particular interest, supply preprints, arrange telephone contacts and find staff to serve as consultants. Noble claimed that this system, and similar programmes at many other universities, including Stanford, had the effect of "subsidizing foreign competition and productivity with taxpayer supported research" in "the interest of a short-term gain".

In a series of frequently interrupted explanations, Paul Gray, president of MIT, fought to play down the importance of the programme, pointing out that all MIT research is eventually published and anyone, whether a member of ILP or not, is free to contact MIT staff.

Congressional subcommittee members found Gray's explanation hard to square with the promises in ILP's advertising brochure and the rewards for participation in the programme given to MIT staff. Ten per cent of the programme revenue is distributed among the staff, with points given for telephone contacts, letters, looking after visitors, going out to factories and laboratories, and so on. And Weiss did not see why US companies should "pay again" for access to research already paid for by their tax dollars.

Eric Bloch, director of the National Science Foundation (NSF), put up a better-received defence of MIT. Bloch argued that basic research results should be freely available; the real problem is not that MIT provides open access to everyone, but that Japan does not provide equal access to its own research. Access is "50 to 1" in favour of the Japanese, he said. Weiss concluded that it might be better for MIT to stop "soliciting" in Japan until problems of access were solved.

Alun Anderson

Tokyo

MEMBERS of the staff of MIT's Tokyo Office repeatedly refused to describe their activities last week, claiming that their director was unavailable for comment. But according to a report prepared in 1986 by the NSF's Tokyo Office, 52 Japanese companies are members of ILP, including such giants as Hitachi, NEC, Mitsubishi and Toyota.

The director of the MIT Tokyo Office is quoted as saying that Japanese companies join the programme because they are "searching for new areas or fields" in which to become involved or because they may "need help and advice" with a new research facility.

The report says that, in 1983–84, 800 people from Japanese member companies attended 25 ILP seminars held in Japan.

Assigning blame before the change

Washington

TROPICAL rain forest may be burning, but Latin American nations do not want to be blamed for the greenhouse effect or other global environmental problems.

Speaking at a forum organized by the Agency for International Development earlier this month, Alvaro Umana, Minister of Natural Resources, Energy and Mines from Costa Rica accused developed nations of making the global environment into "a north-south confrontation" and asked "what moral authority do the developed nations have?" Not very much, according to figures from the Boston-based Conservation Law International and World Resources Institute.

US carbon dioxide emissions totalled 1,200 million tons of carbon in 1985, compared to just 280 million tons for the whole of Latin America. Even Japan produced only a little over 200 million tons, and Britain, West Germany and France all produced slightly less. Of the US emissions, 35 per cent were produced in electricity generation and over a quarter by automobiles. The United States has the lowest gasoline prices and the least efficient automobiles of any of the developed countries.

A Global Warming Prevention Act sponsored by Representative Claudine Schneider (Democrat, Rhode Island) contains a "gas-guzzler" automobile tax among its many energy-saving measures.

Alun Anderson

And, on the average, at least one MIT professor visits Japan each week under the programme.

US companies do not enjoy comparable access to Japanese research because much of the research of interest is done in Japan's private sector. The US-Japan science and technology agreement signed last year is intended to improve US access.

But new Japanese government fellowship programmes introduced in response to the agreement are intended for research in government laboratories and universities. US researchers can carry out research in Japanese companies under NSF programmes, according to Alexander DeAngelis, head of NSF's Tokyo office, but only a handful have taken up the opportunity.

Some US companies are beginning to realize the importance of tapping Japan's excellence in research and development. Notable among them is Eastman Kodak, which last October opened a ¥10,000 million (\$70 million) research and development centre in Japan which is staffed by foreign and Japanese researchers. Such private-sector initiatives may help redress the imbalance in access to research in the two countries.

David Swinbanks

Biomedical bugs

Washington

THE bargain sale of US-financed research data to Japan was not the only conflict of interest to exercise the Human Resources and Intergovernmental Relations subcommittee hearing last week.

Biomedical researchers were seen as particularly likely to suffer conflicting interests, given the huge sums of money from pharmaceutical companies that supplement federal funds for drug development and clinical trials.

Commercialization "has been more aggressive, more brazen, and more experimental than other disciplines", according to Sheldon Krimsky, a professor of urban and environmental policy at Tufts University. He claimed that there is evidence that refusal to share data and biological materials is increasing, even among scientists working for the federal government, implying that "public funds are being used to support proprietary knowledge".

Krimsky believes that new relations between universities and industry, such as Boston University's controversial \$25 million investment in the Seragen biotechnology company, provide a "recipe for conflict of interest". According to his own survey, 45 per cent of Harvard University's biomedical faculty now have formal affiliations with 36 different biotechnology companies.

A.A.

AMAZON FORESTS

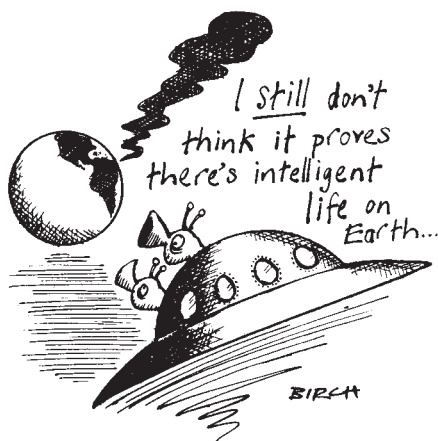
AMAZON EXPLORATION

Burning continues, slightly abated

Sao Paulo

THE dry season is beginning in Amazonia and farmers are getting ready for the *queimadas*, the burning of the forest and savannah-like *cerrado* to make the land fit for pasture and planting. In a new report, the Institute for Space Research estimates the extent of last year's burnings at 121,000 square kilometres in the part of Amazonia south of the Equator.

The figures are less alarming than those released last year by the same team, headed by Alberto Setzer. The total area burned in 1987 was estimated at 204,000 square kilo-



metres, a figure that fueled an international outcry over the destruction of the rain-forest.

Setzer's team used images from the NOAA-9 satellite. He says the statistical reliability of this year's report is greater than last year's because more images were used, 97 this year against 46 last year.

Of the total area burnt, about 40 per cent, or 48,000 square kilometres, was rain forest. According to the report, Mato Grosso was most affected, with 21,700 square kilometres of forest and 27,600 square kilometres of *cerrado* burnt. Setzer said that a record of 8,500 *queimadas* were seen in one day. "Dense smoke... covered areas of millions of square kilometres, bringing health problems to the population, disturbing air, road and river traffic", says the report. Ricardo Bonalume Neto

New posts to come?

Washington

PRESIDENT George Bush's new science advisor, D. Allan Bromley, is trying to entice retiring US National Institutes of Health director James B. Wyngaarden to become deputy science advisor for biomedical affairs. Bromley, a physicist, is seeking to bolster the effectiveness of the Office of Science and Technology Policy, which he oversees (see *Nature* 338, 693; 1989). Bromley is also reported to be considering creating deputy posts for biotechnology and agriculture.

A.A.

Flying raft caught on red tape

São Paulo & Paris

RED tape could delay an unusual scientific expedition to explore the canopy of the Amazon rain forest with a giant platform suspended from a hot-air dirigible. The project, directed by Professor Francis Hallé, of the Botanical Institute at the University of Montpellier II in France, was due to start on 1 July. But the researchers, who have been waiting since May for official clearance, could face a further delay of four months while their application passes through the appropriate diplomatic channels.

According to Hallé, the 'canopy raft' (*radeau des cimes*) provides a unique opportunity to study the rich flora and fauna of the rain forest, hitherto little explored because of its inaccessibility. "Most of the selective forces operate 30 to 50 metres above the ground", he says. This makes access for teams of scientists extremely difficult. All of the previous methods of access, such as trunk-climbing, fixed towers, aerial walkways and remote aerial photography have disadvantages. Only a few tree crowns can be studied, making it impossible to obtain statistically valid samples or to carry out team observations.

The 'canopy raft' is the third in a series of attempts by Hallé and his colleagues to land structures on forest canopy.

The idea is that a light structure comprising rubber inflatable 'sausages' joined together by a net allows a small team of researchers to land on the tree tops where they can stay as long as they wish, exploring a relatively wide area before moving to another site. The first prototype, built in 1985, was a simple triangular structure lowered by helicopter in forests in central France. But the helicopter rotors damaged

trees and so a second version, tested in French Guyana in 1986, used a hot-air balloon.

The latest canopy raft is a more complex hexagonal structure with a 700-square-metre platform of netting held together by a soft frame and displaced by a hot-air dirigible made by the UK company Thunder and Colt. The raft was designed by Gilles Ebersolt in consultation with its pilot, Danny Cleyet-Marrel.

After an initial flight over a section of the Brazilian Amazonian forest to select sites, the platform will be lowered onto the canopy. From here the small team, including botanists, Hallé, Patrick Blanc and Isabelle Valade, can descend using rope ladders or explore different areas of the tree crowns, sleeping overnight in a shelter on the platform.

The project will also involve large-format stereo photography and radiometric measurements.

The project has been financed by the European Economic Community and a number of private sponsors, mostly Japanese. The involvement of twenty researchers at the Brazilian National Institute for Amazon Research (INPA) and about 15 from the University of São Paulo should help the study to obtain authorization. But according to Guilherme Euclides Brandã, of the National council for scientific and technological development, the French team's visa application was received only at the end of May.

Permission still has to be obtained from the Brazilian Institute of the Environment and Renewable Resources and, possibly, from the secretary for national defence counselling. This, says Brandão, could take several months.

Peter Coles & Ricardo Bonalume Neto

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

Canopy raft to be used by French researchers to study the Amazon rain forest canopy.

Researchers still at odds

Washington & Boston

ALTHOUGH the greenhouse effect can be blamed for making environmentalists out of many of the world's leaders, including both US President George Bush and French President François Mitterrand, many researchers are still not convinced that there is a link between gases accumulating in the atmosphere and observed global warming.

Disagreement about current climate models was apparent last week at a Harvard University seminar on "Understanding global warming". James Hansen, chief of the NASA Goddard Institute for Space Studies claimed a "high degree of confidence" in the cause and effect relationship between the greenhouse effect and observed climate change. But Thomas Karl of the National Climatic Data Center has a "small degree of confidence in the link". And Ron Prinn, professor of meteorology at Massachusetts Institute of Technology, takes the middle way, describing the link as a "quite plausible hypothesis".

The uncertainties in the models are large, says Karl, and so are the temperature data collected over the past century. He pointed out that the time of day at which temperatures are taken has sometimes changed, as have the kinds of shelters in which thermometers are housed. And monitoring stations have moved from city centres to suburban airports.

A report* issued last week by the George C. Marshall Institute, a Washington-based think tank, adds to the doubts. It says that solar variability might provide a better explanation for the 100-year rise in mean temperature than increased concentrations of carbon dioxide. If that is correct, then only a small greenhouse effect might be expected in the next century.

The report's authors are Frederick Seitz, Robert Jastrow and William Nierenberg — former heads of the National Academy of Sciences, the NASA Goddard Institute for Space Studies and the Scripps Institution of Oceanography. Before policy-makers begin corrective programmes, the authors argue that the uncertainties of greenhouse forecasts must be reduced.

They advocate a \$100-million investment in advanced supercomputing facilities to do the job. Bigger computers would allow a better treatment of the role of oceans and clouds in modulating the greenhouse effect and provide fine-scale predictions of regional changes in climate.

Seth Shulman & Alun Anderson

*Scientific perspectives on the Greenhouse Problem, George C. Marshall Institute, Washington DC.

Changing with the fashion

Washington

ENVIRONMENTALISM is all the rage in the United States, and even long-time opponents of the conservation movement are finding it pays to change views. In Washington, power-company executives are seen holding press conferences with energy-conservation lobbyists, Congressman from the industrial mid-Western states queue up to support greenhouse-gas legislation and President George Bush is praising the environmentalists who for the past eight years were not permitted past the White House gates.

Bush's kind words for the environmentalist movement came last week as he announced proposals to "curb acid rain and cut urban smog and clean up air toxics" through a new clean air act. But the kind of environmentalism that is finding favour with Bush and his friends in industry has a new slant, substituting the power of market forces for moral outrage and blanket control measures.

The new approach has been heard of before, but did not emerge as a comprehensive philosophy until the publication of "Project 88, Harnessing Market Forces to Protect our Environment", a report drafted by Robert Stavins, professor at the Kennedy School of Government at Harvard University. Last week, an impressive array of representatives of the president, the Environmental Protection Agency, conservation groups, congressmen and academics turned out to endorse its approach.

Project 88's ideas have already had an impact on Bush's clean air act proposals. Bush wants "to end acid rain by the end of the century" by cutting sulphur dioxide emissions by almost half, along with cuts in nitrogen oxide. Federal regulators' first impulse on hearing that sort of news is to rush for a new set of tough emission standards for all power plants. But Bush intends to follow Project 88 and set only total output limits and then leave private industry to decide how to meet them by trading pollution credits among themselves. An electric power company may decide to earn credits by switching to low-sulphur coal or installing expensive scrubbers. Or it may find it less expensive to buy pollution credits from other plants that can cut pollution more easily.

The result should be that industry as a whole is under constant pressure to find the cheapest and easiest way to cut total pollution levels. New plants will probably meet high standards easily, gaining credits, while old plants is more likely to be covered by buying pollution credits rather than by expensive alterations. Under the old "command and control" system every plant would have to meet the same standard, regardless of cost. The

idea has already been tried out in a limited way and, according to EPA figures, is saving \$500,000 a year. The EPA says that full implementation will save \$3,000 million a year by 1995 over uniform emission standards designed to produce the same effect.

The Canadian government, which sees much of the US sulphur dioxide emissions end as acid rain on its territory, is pleased with the proposed reductions and also interested in the concept of making market forces drive pollution reduction. If the people rushing to endorse Project 88 are right, the idea has wide application. Project 88 lists 36 possible new policies; among them is a deposit-refund scheme, just like that used to encourage people to recycle bottles, that could end toxic dumping. The idea is for toxic chemicals to be sold with a deposit that would be returned when the waste was turned in at a facility for recycling or disposal.

The new trend to make conservation just another place to do business worries some environmentalists who see it as slippery slope that bypasses moral sanctions and leads to weaker policies. But it is already proving a success in New England where two long-time adversaries, the Conservation Law Foundation and Northeast Utilities, a large electric power company, decided last year to end five years of litigation and start collaborating. The result is a scheme in which a group of power companies are paying their customers to use less electricity by giving them energy-efficient equipment. Although power-company accountants may not at first be happy with the idea that reducing demand is good for profits, when conservation means that the utility need not spend millions on a new plant, the idea looks very attractive. Essentially, the power company makes money by buying efficiency improvements that save power at less the total cost of generating it.

If such big savings can be made, why do people not buy energy-efficient equipment without help from power companies? The answer is that savings are made over a long period, and people do not have the long-term perspective of power companies. The scheme was assessed at a World Resources Institute meeting earlier in the month by Doug Foy, President of the Conservation Law Foundation and William Ellis, president of Northeast Utilities. Ellis charted his road to support for energy conservation as having passed through "disbelief and denial, then skepticism and grudging acceptance". The two said that New England power companies will spend \$100 million on energy-efficiency programmes this year. By 1995 they will spend \$500 million a year.

Alun Anderson

Old tasks given a new life

Paris

"If we work for the poor and the penguins simultaneously, we can hope for a better common present and future", says Professor M. Swaminathan, President of the Indian National Academy of Sciences. His poetic statement gives the gist of a meeting at UNESCO, the United Nations Educational, Scientific and Cultural Organisation, last week*, involving about 90 scientists and heads of multilateral science and technology organizations.

The meeting followed another Paris colloquium on global environment issues, "*Planète Terre*" (see right), and several participants attended both meetings — not a coincidence, according to a UNESCO spokesman, but "smart timing". UNESCO's aim, said director-general Federico Mayor, was to encourage "the international community as a whole to take a fresh look at the status of cooperation in science and technology on the eve of the closing decade of this century."

At the end of August, the United Nations Intergovernmental Committee on Science and Technology for Development (UNCSTD) will meet in New York to evaluate progress since its meeting in Vienna in 1979. But, Mayor emphasized, the Paris meeting was not intended to upstage that end-of-decade review, simply to "spark off new dialogue".

Many of the participants agreed that actions proposed in 1979 have failed to materialize. "There was total agreement at UNCSTD", according to Dr M. G. K. Menon, president of the International Council of Scientific Unions, "but there was no follow-up, there was no implementation, there was no adherence by nations to the decisions they were party to."

This, said Sergio Trindade, Assistant secretary-general of UNCSTD, had come about because priorities have been mixed; one result has been that organizations have been supported, but goals have not been set. Faced with the "sclerosis" of the Vienna proposals, Menon says the need now is for a "collateral bypass operation" to strengthen endogenous science in developing nations, while creating better international collaboration.

Many agreed that UNESCO, the only United Nations organization specifically concerned with science and technology as well as education, would be ideally suited to perform this 'operation'. But this is unrealistic so long as Britain and the United States continue to refuse to rejoin.

According to F. Sagasti of the World Bank and chairman of the UN advisory committee for the application of science and technology to development, all of the

multilateral organizations need to be reshaped. They were set up "in the shadow of East-West conflict", he says, whereas the present and future dialogue must be between north and south.

But Professor Roald Sagdeev of the Soviet Academy of Sciences felt that technology cooperation between East and West still has far to go. When the University of Chicago sent an experiment aboard the Soviet probe in the encounter with Comet Halley in 1986, a 'black box' of Radio Shack components was used to overcome COCOM regulations on technology transfer to Eastern bloc countries.

The main obstacle to north-south collaboration is the widening rift between the 1.1 billion haves and the 3.6 billion have-nots, said Professor Abdus Salam, director of the International Centre for Theoretical Physics (ICTP) and President of the Third World Academy. Developing countries still see basic science as a luxury and, as a result, lack the trained teachers, engineers and scientists to collaborate. Salam argued that more institutes on the lines of ICTP are needed and has launched an appeal to create three new international centres — for high technology and new materials, for pure and applied chemistry and for earth sciences.

The conference lacked neither realism nor optimism in its analysis of the future of science and technology for development. Much now hangs on the outcome of the UNCSTD meeting and, eventually, on the return of the United States and Britain to UNESCO.

Peter Coles

France promises timely action

Paris

ONE hundred and eighty scientists from 40 countries met in Paris last week to discuss the Earth's changing climate. The conference, called "*Planète Terre*", was organized by French research minister Hubert Curien, under the patronage of President Mitterrand. It followed on from the international meeting of politicians at The Hague last March to discuss the depletion of the ozone layer.

The aims of the meeting were twofold: to recognize the extreme complexity of global interactions from the upper atmosphere down to the Earth's core; and to provide decision-makers with a more realistic idea of what climate changes might occur and what might be done about them.

The scientific organizer of the conference, Claude Allègre, said that action would raise delicate problems because "some issues need rapid decisions, but science is not always that certain". More research is needed quickly, on a global scale.

In his closing address, Mitterrand insisted on the need to share knowledge across frontiers and to develop more cooperation between Northern and Southern Hemispheres. He also used the opportunity to clarify France's position on the annex to the Antarctic Treaty, which would control mineral exploration. Both France and Australia have said they would not sign the new annex. Mitterrand said he had been "seduced" by an idea put forward by Commander Jacques Cousteau, the marine biologist, to turn the Antarctic into a nature reserve.

Peter Coles



The completion of the large electron-positron collider (LEP) was announced last week by CERN, the European laboratory for particle physics. The circular machine, housed in a 27-km-long tunnel between 50 and 150 m below the surface, accelerates electrons and positrons to a combined energy of 200 GeV. LEP's main purpose is to investigate the Z^0 particle, discovered at CERN, and although the Stanford linear collider recently made its first Z^0 (*Nature* 338, 606; 20 April 1989), LEP has more than two times the beam energy of its rival and is expected to generate Z^0 s at a higher rate. LEP is the result of an eight-year project financed by the 14 member states comprising CERN. □

* The High-Level Colloquium on Science and Technology for the Future: A Fresh Look at International Cooperation in Science and Technology.

Thirty-year secret revealed

London

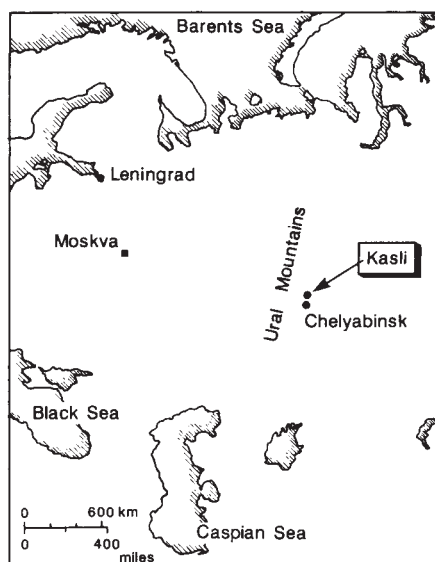
THIRTY-TWO years after the event, the Soviet news agency Tass last week carried a report of the nuclear explosion at Kasli, near Chelyabinsk in the Urals, in September 1957. This is the first official disclosure of the accident — although, by 1961, West European and US defence circles were well aware that some such incident had occurred.

As far as the world scientific community was concerned, it was Dr Lev Tumerman (in *Nature*) and Dr Zhores Medvedev (in his book *Nuclear Disaster in the Urals*) who, in the 1970s, first drew attention to the disaster and its aftermath. Much of the evidence was based on discreet reports in Soviet journals about the dispersion of radioactivity by migratory birds.

According to Tass, the details of the explosion have been revealed by Boris Nikipelov, the first deputy minister of "Medium Machine Building" — the cover name for the ministry responsible for both the civil and military nuclear industries. The incident, he said, was a chemical explosion in a tank containing radioactive wastes. As a result, a radioactive 'trail' was formed, 105 km long and 8–9 km wide. The discharge was about 2 million curies, Nikipelov said, that is, 0.04 times that of Chernobyl. There were no casualties, but 10,000 people had been "urgently

evacuated" from the contaminated zone.

Because the waste tank had been sited at a facility for the development of nuclear weapons, he said, the disaster had never been reported in the press. By 1978, "economic activity" had been restored to over 80 per cent of the contaminated zone, he said, and the rest had been converted into a "reserve". For the past 15 years, the



radiation situation had been assessed by experts as "safe".

Nikipelov's revelations have been made as a response to a campaign in Chelyabinsk against a proposed nuclear power station. Nikipelov admitted that some water reservoirs in the area had been contaminated by radioactivity since 1957, but said that the choice of the area for a power station was "not accidental". The power station, he said, would evaporate the water, thus purifying the reservoirs. Moreover, a commission of independent experts is analysing the safety of the proposed nuclear station and the site's suitability.

This application of *glasnost* to the Urals disaster has come when there is increasing concern about the Soviet Union's safety record. Since the lifting of reporting restrictions on accidents three years ago, the Soviet media have reported a long sequence of civil and military disasters on land, sea and air.

During the past few months, however, there has been growing criticism of the Soviet record in rescue work. Why, the new generation of investigative reporters ask, does the Soviet Union not have the life-saving facilities available in the West?

Following the disaster on the Trans-Siberian railway earlier this month, the weekly *Literaturnaya Gazeta* published an interview with the Rector of the Baskkir Medical Institute, who advocated the establishment of special training courses in post-disaster rescue work. A few weeks

Japan's blue sky grows even bigger

Tokyo

IMAGING and manipulation of materials at the sub-micrometre to atomic level feature in three new ERATO (Exploratory Research for Advanced Technology) projects just announced by the Research and Development Corporation of Japan.

ERATO, which is funded by the Science and Technology Agency, supports out-of-the-ordinary research by joint teams of young researchers from universities and industry. It received high praise in a recent assessment by the US National Science Foundation. Each project runs for 5 years with a budget of \$2–3 million per year.

Akira Tonomura of Hitachi Central Research Laboratory near Tokyo heads the "electron wavefront" project which will observe materials and magnetic fields in three dimensions at the sub-micrometre level using electron holography. Tonomura's group recently succeeded in holographic imaging of tiny magnetic fields (fluxoid quanta) attached to superconductors. The research is expected to contribute to development of next-generation high-speed electronic devices, such as quantum flux parametrons (another subject of study under ERATO).

Masakazu Aono of the Institute of Physical and Chemical Research continues ERATO's taste for exotic project titles with his "atomcraft" project, also intended to help develop new electronic devices. Using a scanning tunnelling microscope, his team will try to create new semiconductor materials by implanting individual atoms on the surface of materials. And Joh-E Ikeda of the National Institute of Agrobiological Resources will head the "genosphere" project to observe the movement and distribution of chromosomes in living cells with the aim of understanding genetic disease and ageing. David Swinbanks

earlier, Gavrill Popov, newly elected to the Congress of People's Deputies as representative of the Union of Scientists' and Inventors Societies, had castigated the Soviet authorities in connection with the loss of the submarine *Komsomolets* and for valuing the hardware above the lives of those who operate it. Humanitarian considerations apart, he said, this is also uneconomic, given the cost of training personnel.

There are now fears that the various commissions appointed to investigate the causes of accidents are programmed to concentrate on human error rather than to consider safety procedures themselves. Yet, according to an article in the transport-workers journal *Sredneazitskaya mequstral* (Central-Asian trunk route), current operational and safety instructions are such that they could, in reality, lead to a "fire by the rule book". Vera Rich

RESEARCH IN INDIA

New centre for theoretical research

New Delhi

A CENTRE for advanced scientific research is to be set up in Bangalore and named after Jawaharlal Nehru, India's first prime minister, whose centenary is being celebrated this year. The \$16 million centre will be structured along the lines of the international institute for theoretical physics at Trieste in Italy.

Professor C N R Rao, director of the Indian Institute of Science (IIS), also in Bangalore, is to act as president of the Nehru centre which, he says, "will be devoted to scientific research at the highest level in frontier areas". It will set up its own facilities for theoretical work and will have access to the workshop and laboratories of IIS for experimental work.

The centre has been registered as a non-profit society with the primary objective of providing an excellent climate for research and acting as a national and international forum for free exchange of ideas through workshops, seminars and summer schools. It will have a full-time faculty as well as honorary and visiting positions. Rao hopes the new centre will attract Indian intellectuals now drawn to Trieste.

K. S. Jayaraman

HUMAN FRONTIERS SCIENCE

Management issues settled

Tokyo

AN international council of scientists will have the biggest say in selecting the research objectives of Japan's Human Frontier Science Program. That is the most important outcome of discussions of the programme held last week in Tokyo by government representatives of the seven Western summit nations (Japan, Canada, France, West Germany, Italy, Britain and the United States) and the European Community.

Debate over the organizational structure of the programme was "heated", according to Toichi Sakata, director of the programme office at the Science and Technology Agency. But in the end, representatives accepted Japan's proposal that there should be a governing board of trustees consisting of government representatives to set basic policy and make final management decisions, an international council of scientists to set research objectives and organize a peer-review system for the award of grants and fellowships and a secretariat of administrators drawn from the summit nations. A particularly contentious issue was that of whether the board of trustees can override decisions of the council of scientists.

Finally, it was also agreed that "purely scientific decisions" by the council — for example, selection of research themes within the areas to be covered by the programme (the brain and molecular recognition and response) — cannot be overridden by the board, Sakata says. It was also decided at the meeting that, as the programme is developed, the council will be able to change research priorities and topics and initiate new lines of research provided that the board agrees.

The secretariat will be located in an office in Europe due to open this October, but the site is not yet decided. Three locations are under consideration: London, Strasbourg and Rome. The highly political decision on location will be made by consensus among the summit nations by late July or August when the final meeting to set up the Frontiers Program will be held, Sakata says.

The Science and Technology Agency and the Ministry of International Trade and Industry have a total of about \$20 million for the programme in this fiscal year (ending in March 1990). Twenty 3-year grants of \$0.5 million each and 100 2-year postdoctoral fellowships will be awarded to international teams of scientists from the summit nations. Ten workshops will also be held by the end of March. Solicitations for grant and fellowship applications will begin in August.

David Swinbanks

WHALING

Japan contemplates bigger kill

Washington

AN attempt by Japan to double the number of whales it will be allowed to kill this year failed when the International Whaling Commission (IWC) refused last week to consider a request for permission to kill whales in Japanese coastal waters. Japan asked that its traditional coastal whaling communities be exempt from the moratorium on commercial whaling on the grounds that Alaskan eskimos are already granted a similar exemption.

But the IWC postponed discussion of Japan's request to its next meeting, to be held in 1990 in The Netherlands. The moratorium will then be reconsidered in the light of the assessment of world whale populations now under way.

Japan was also refused a request for an interim allocation of 320 minke whales, which infuriated the Japanese representative, who said that more porpoises and small cetaceans would be killed instead. IWC members are divided over whether these animals should be within the council's remit, with Latin American countries arguing against it on the grounds that it would interfere with their sovereignty.

For the third consecutive year, the killing of whales for "scientific research" by Japan, Iceland and Norway was criticized by the IWC, although in language toned down from previous years: instead of asking governments not to grant permits for "scientific" whaling, IWC asks merely that they "reconsider".

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

Greenpeace/Morgan

Minke whale fishing on the deck of the Japanese ship Nisshin Maru 3.

ANIMAL RIGHTS PROTESTS

French researchers plan to hit back

Paris

RESEARCHERS from the Lyons laboratories raided by animal-rights activists last month (see *Nature* 339, 407; 8 June 1989) have decided to create an association to defend animal experimentation. The move follows what a communique describes as "a campaign of misinformation" in the media over the past few weeks.

Researchers, says the communique, are being portrayed as "Frankensteins", carrying out "cruel and senseless experiments". To correct this view, the association hopes to inform the press of the strict controls imposed by law to minimize animal suffering and to illustrate the importance of using animal models to understand fatal diseases such as cancer, Parkinson's disease and Alzheimer's disease. The association has invited a number of "prestigious personalities" and researchers to meet the press this week.

Meanwhile, a number of animal-rights groups stood in last weekend's European elections. One of their electoral bids is to "create an ethics committee to fight against vivisection and animal experimentation". Although they gained no seats in the European Parliament, they received about 180,000 votes.

Peter Coles

IWC adds that the only research that has any value in assessing and conserving the whale populations is that in which whales are not killed. But ignoring the criticisms, each of the countries concerned announced plans for more scientific research. Japan will take 400 minke whales this year, up from the previous 250. Norway will take 20 minke whales and Iceland will take 68 fin whales. The proposed kills were condemned by the IWC.

The World Wildlife Fund is now taking legal action against the United States for not imposing fishing sanctions against Iceland. Under the amendment, the United States can impose sanctions against countries failing to heed IWC limits.

New data on whale populations were presented at the meeting last week based on sightings south of latitude 60° S between 1978 and 1984. The blue whale population is now estimated at 200–1,100, compared with the 1965 estimate of 11,000.

The new estimate surprised the IWC scientific committee but a spokesman said that an explanation of the discrepancy will have to wait full analysis of the new data. The new estimate is based on sightings whereas previous estimates have been based on whalers' kill reports. An expected fin whale population of 100,000 was estimated at only about 2,000 but the spokesman said a large proportion of the fin whale population may be north of the area surveyed.

Christine McGourty

Sharing astronomical costs

SIR—Astronomical discoveries in the past few decades have transformed our view of the Universe and what it contains. Radiation has been detected that originated in the early moments of the Universe, as well as from massive black holes, pulsing neutron stars and distant, incredibly energetic, quasistellar objects. The price of these discoveries has become increasingly high, reaching billions of dollars for the most powerful new telescopes in space.

Cooperative arrangements have been established between our countries in several areas for specific one-time-only scientific experiments and have been extremely successful. In addition, the Soviet Union and the United States have participated in *ad hoc* collaborations involving individual scientific groups in many different nations. In fact, in space astronomy, international experiments are now the rule rather than the exception. Ground-based astronomy could also benefit from the mutual stimulation and pooling of resources that result from such joint efforts.

We propose the establishment of an international institute for astrophysics to be organized by scientists, administrators and statesmen from the Soviet Union, the United States and from any other countries that wish to participate scientifically and financially. The joint goals of this institute would be to promote astrophysical research and to foster scientific understanding. The details of the operation and scientific programme of the institute would have to be decided on the basis of discussions among a wide range of the relevant people in the interested countries.

We mention below one exciting scientific possibility, currently beyond the ability of any one country to achieve, that serves as an example of what we have in mind. We stress that extensive discussions are required in order to choose among the large number of important projects in ground-based or in space astronomy that might become focus of the institute. The institute might begin with the long-term goal of placing a 10-metre optical and ultraviolet telescope in space by the year 2010, perhaps in geosynchronous or high-apogee orbit. This Large Space Telescope would make possible unprecedented studies of the most distant objects in the Universe as well as revealing important new facts about our astronomical neighbours. As a first step, the institute could use available resources to place in geosynchronous orbit, with appropriate modern instrumentation, the 2.5-metre test mirror developed in connection with the Hubble Space Telescope programme. These two projects give the flavour of what is possible with such an institute.

The physical location of the institute

would have to be decided by an international group that would take into account the relevant scientific and political factors. The staff would be international and the facilities would be available to all scientists of participating countries. In the first few years of its existence, the institute scientists could develop, combine and exploit the capabilities of archival research based upon existing data banks.

We call upon our colleagues and political leaders to consider the viability and desirability of this proposal.

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CSIRO cuts

SIR—The report of increased support for Australian science and technology (*Nature* 339, 167; 1989) is splendid news for Australian scientists and their colleagues overseas.

It is much less good news that in the same article it is reported that the Commonwealth and Industrial Research Organisation (CSIRO) will suffer further cuts to be added to those of recent years. These cuts arise partly from the current fashion, all too familiar in Britain, that government-supported scientific institutions should concentrate on market-orientated research and generate their own support from the private sector. This was coupled with an almost unbelievable administrative bungle, mentioned in your article, in which a large sum estimated to be coming from private industry did not materialize.

The consequences of this mismanagement are now to be inflicted on CSIRO scientists by job losses and budget cuts. These combined pressures are having disastrous effects on morale and on the work done by research groups with international reputations. For example it was astonishing to learn at a recent meeting in Southampton on marsupials as models for developmental studies, that the group led by C. H. Tyndale-Biscoe in Canberra, which has an international reputation in reproductive biology as a result of its

extensive studies of one of the very few established experimental colonies of larger marsupials (tammar wallabies), is seriously considering abandoning its marsupial research to concentrate on developing new biological methods for control of rabbit and fox populations.

The explanation is that funds are not available for both the fundamental work on marsupials and the more applied studies, and that there is pressure on the group to do the latter. If marsupial studies are abandoned, the world will lose a resource and an expertise that will not easily be replaced. The Australian government should ensure that such a loss does not result from either its short-term policy on 'market-orientated research' or from bureaucratic incompetence.

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Reprint requests

SIR—I agree with Mathew T. Martin-Iverson (*Nature* 337, 594; 1989) and Ivor Smith (*Nature* 336, 708; 1988) that many scientists request reprints on the basis of the title of an article without actually reading the article. The pros and cons of requesting reprints have been discussed by the two authors. In a recent study, I sent out a questionnaire to each of the 457 individuals who requested a reprint from me to determine how and why the reprint requests were made (*Am. J. Dis. Children* 143, 121–123; 1989). There were 259 replies available for analysis. Only 44 individuals (17 per cent) had read my article (or abstract) before the request was made. One individual (0.4 per cent) did not answer the question. The remaining 214 (82.6 per cent) confirmed that they had not read the article (or abstract) before the reprint request was made. The reasons for requesting reprints were: journal not available locally (156 responses), photocopying facilities not available locally (8), photocopying prohibited by copyright regulations (7), high quality of the reprint (95), local institution would pay for the mailing of the reprint cards or letters but not for the photocopying (14), library not readily accessible (8), photocopying time-consuming (8), personal contact with the author (1) and space-saving for reprint libraries (8).

It must be remembered that reprints are costly to authors and reprints should not be requested unless there is a really good reason for it.

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Japanese universities feel the chill

Akio Yamamoto

Although Japan is now regarded as a rich country, its support for academic research is disproportionately meagre. Researchers themselves may have to provide the impetus for change.

VISITORS from abroad are often surprised by the contrast between the substandard conditions in Japanese university laboratories and the well-equipped laboratories in private industry. Japan is now regarded as rich and successful in producing high-quality commercial products, but university researchers have not benefited from the remarkable increase of Japan's GNP. Rather than complaining, my colleagues and I have attempted to draw attention to the situation by organizing a committee of members of the Chemical Society of Japan to examine how university chemistry departments are financed. Our report, which has recently been published (see ref. 1), reveals inadequate conditions, and we believe that our conclusions reflect conditions in fields other than chemistry — apart from those devoted to 'big' science — in Japanese universities.

It may be hard for some to believe that Japan's university researchers are in this predicament when the GNP is growing, but most university researchers will agree with our report. The total amount of expenditure for research and development in Japan soared from ¥0.43 million million in 1965 to ¥7.2 million million (\$58,000 million), corresponding to 2.6 per cent of the GNP in 1986 (Fig. 1). The increase largely results from the expansion of research and development by the private sector; government investment in universities has risen only modestly. The relative share of government support for

research and development has declined from 30 to less than 20 per cent in the past 20 years.

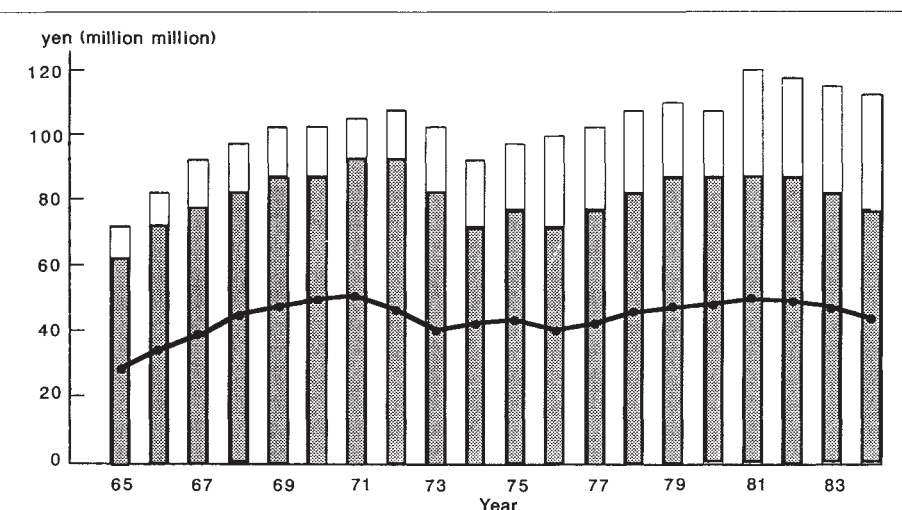
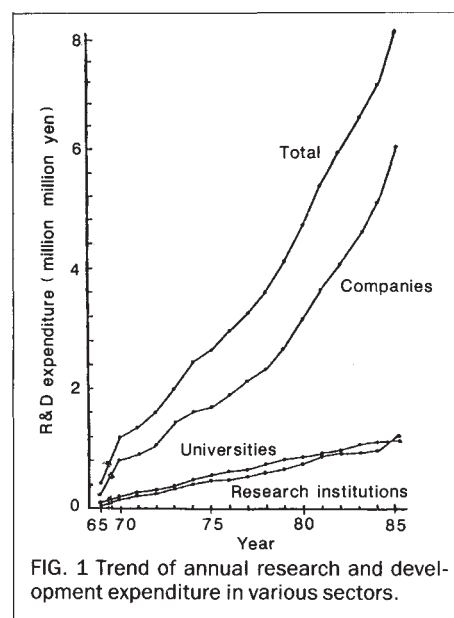
During this period, the number of researchers both in industry and in universities increased considerably. The amount of money spent per person for research and development in companies increased from ¥14 million to more than ¥20 million, whereas that for university researchers dropped from ¥10 million to ¥8 million (on a 1980 yen basis). In fact, conditions for university researchers are even worse than these figures indicate, because the ratio of salaries to total research expenditure is greater in universities than it is in companies. Although university researchers are generally paid less than those in industry, their salaries still cut into the rest of the research budget.

The ratio of technical staff to researchers decreased from 0.85 in 1965 to 0.31 in 1985. The decrease in technical support means a drop in efficiency. Japanese high-tech industry is renowned for its efficiency but in the universities, where young researchers and students spend more time doing chores that could be done by less qualified people, this is not the case.

I will next examine the money spent on each researcher in universities. First, a brief explanation of the research system may be useful. There are three main sources of research money. The first is the funds given annually to each research unit.

In the older national universities, including seven former imperial universities, each unit, called a *koza* (teaching chair), consists of a professor, who chooses the direction of research, one assistant professor and two research assistants. In the newer national universities, there may be only one research assistant. The fixed amount of money annually provided to each *koza* comes from the Ministry of Education, Science and Culture, and can be spent on chemicals, small research equipment and other similar items. This corresponds to the general university fund in Britain. In private and other municipal universities, money is distributed by the administration of each university. Although there are many private universities, they have so far made a relatively minor contribution to science and technology. Trends can be seen more clearly by analysing the 100 or so national universities. The general university fund given annually to the national universities has stayed relatively constant for the past 20 years (Fig. 2). During this period, the number of researchers almost tripled. As a result of this, the amount given to each *koza* has decreased by about half since 1964, once inflation is taken into account (Fig. 3).

The second source of money is the Scientific Research Grant, which comes from the national budget. The money is distributed by a peer-review system and supports basic and applied research in



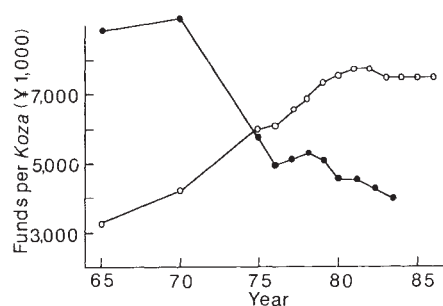


FIG. 3 Trend of general university funds given to each koza. Open circles, nominal terms; closed circles, real terms.

universities. The size of the annual national budget for this category has trebled in the past 20 years in real terms, reaching ¥50 thousand million this year. (This figure is dwarfed by the amount spent by industry on its own research. Hitachi, for example, spent ¥300 thousand million on research and development last year.) Because of the steady increase in the number of researchers, however, the number of total applications for these grants also increased, so that only one grant application in four or five is successful.

The third main source of research money in the universities is from industry. This has kept rising in the past 10 years to about half of the Scientific Research Grant. The donation from industry has improved the funding situation in technology-oriented upper-rank universities, but has not helped many poorly funded universities.

Because of the gradual relative decrease of the general fund, researchers have come to depend more on the Scientific Research Grant. This has resulted in growing frustration of people in provincial universities, who have not had much success in obtaining money via the Scientific Research Grant.

Our survey of chemistry indicates that researchers are dissatisfied about research funds and equipment (Fig. 4). The lack of money is most keenly felt in applying for relatively expensive equipment and in obtaining laboratory space. About 85 per cent answered that expensive equipment and space are insufficient or extremely deficient. The degree of dissatisfaction is much higher than that of researchers in the United States; a National Science Foundation survey found that about 40 per cent felt that the research equipment is insufficient, whereas 50 per cent thought it adequate and 10 per cent excellent².

Despite the inadequate government support for university researchers, the contribution of Japanese researchers to scientific journals has steadily increased, at least in number, to the extent that it arouses uneasiness among US researchers (see Fig. 5)³. Although the number of Japanese Nobel prizewinners in science is so far small, other yardsticks, such as citation indices, show the improvement in

scientific standards of fundamental research on almost every front with respect to international competition, despite poor support from the Japanese government.

There may be various factors associated with the rise in basic research activity, such as hard work, the relatively high level in the quality of researchers, effectiveness of the funding system and effectiveness of the research system. I shall now consider the last two factors.

It is generally agreed that the dual funding system in Japan, composed of the general fund and the peer-review system, has been effective in sustaining the level of long-range research activity and in promoting good research projects. On the other hand, opinion is divided about the merits of the *koza* system. Its advantage is that the average size of a unit is suitable for pursuing several research projects in a manageable fashion. The system helps the younger researchers, who are guaranteed the essential research conditions and do not have to spend much time in applying for grants. These researchers usually collaborate with graduate and senior undergraduate students, which provides reasonable manpower. The disadvantage of the *koza* system is that the younger researchers have less independence than in the United States. In particular, brilliant individuals may find it difficult to try out their own ideas, especially when they are supervised by mediocre, unsympathetic professors. The *koza* system is sometimes blamed for the disproportionately few Nobel prizewinners in Japan, as Dr Susumu Tonegawa, one of the few Japanese Nobel laureates, who works at MIT in the United States, has pointed out. But the *koza* system has been reasonably effective in

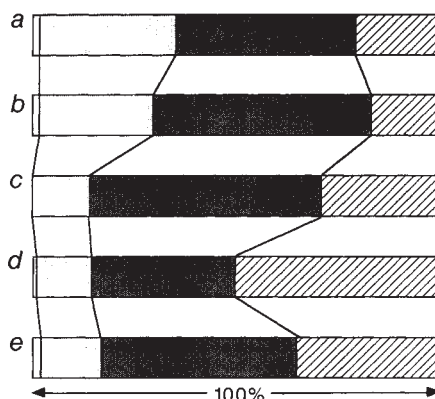


FIG. 4 Degree of satisfaction of chemistry researchers about conditions. Far left, sufficient; next on the left, not sufficient but manageable; dark shading, insufficient; diagonal stripes, extremely deficient. a, Fund to buy small equipment and chemicals less than 1 million yen; b, equipment costing more than 1 million and less than 3 million yen; c, more than 3 million and less than 10 million yen; d, more than 10 million yen; e, space, buildings and facilities.

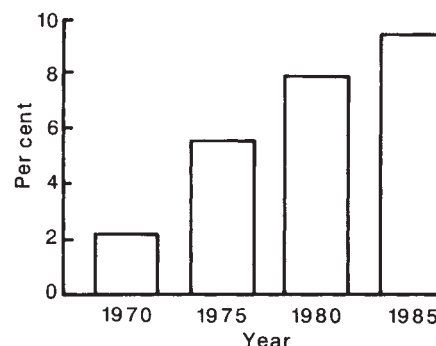


FIG. 5 Contribution of Japanese chemists (in per cent) to the *Journal of the American Chemical Society*.

sustaining research activity. Younger researchers might find it easier if there were more mobility between universities at each stage of their career.

The other shortcomings in the Japanese funding system are restrictions arising from the single-year budgetary system, and from the fact that money for hiring researchers is not included. Thus, even very successful investigators cannot usually hire postdoctoral fellows from their own research fund, and find it difficult to employ people to work on important projects where there is intense competition from abroad.

On the other hand, in a funding system that does not include stipends, a relatively small sum of money can quickly improve the standard of equipment and the efficiency of research. This was dramatically demonstrated in 1987 when the government provided extra money for expensive equipment to boost domestic demand, in the face of pressure from Western countries to improve the trade imbalance. The amount of money provided to universities via this special budget accounted for only about 1 per cent of the total money spent, but it was used effectively to upgrade outdated facilities.

Having realized the importance of basic research, Japan must now contribute to the progress of science using its economic power and its relatively large number of trained researchers. It is ridiculous that, despite its postwar economic boom, Japan has been accused of freeloading on Western science. It is obvious that increased investment in basic research will be in the national interest. Changing the attitudes of administrators in the Ministry of Finance will take time, but it is worth our sustained effort. □

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Towards the calculation of DNA

A remarkable calculation of the vibrational motion of DNA, valuable in its own right, may be most significant as an inspiration to those who believe that realistic calculations of DNA are within sight.

ONE of these days, somebody will begin a paper by saying, in effect, "Here is the hamiltonian of the DNA molecule" and will then, after a little algebra, explain just why it is that the start and stop codons of the genetic code have their precise functions, or how polymerase molecules work in the process of transcription. That particular paper, destined to be among the most often cited in the year of its publication, has not yet been written. But there is now a paper beginning in that vein even if its authors, M. Peyrard from the University of Dijon and A.R. Bishop from the Los Alamos National Laboratory, modestly disclaim grand pretensions for their work (*Phys. Rev. Lett.* **62**, 2755; 1989).

Peyrard and Bishop set themselves the goal of understanding the denaturation (or strand-separation) that is a part of the process of transcription. Theirs is not the first attempt to calculate something of the process, but most previous attempts have started with the observation that the dynamics of the familiar double helix must be nonlinear, and have set out to tell whether soliton waves might account for a pulse of local denaturation moving along the helix at some steady rate.

The new effort is interesting and important because it is a more general approach. Starting with a model of DNA, Peyrard and Bishop set out to calculate its partition function, both as a way of deriving the thermodynamic properties of DNA and also of calculating the likelihood of fluctuations at particular parts of the molecule, denaturation pulses, for example. It is remarkable that it has been possible to carry the calculations through without having to resort to numerical calculations, even if the price paid is a model of DNA which is much simpler than reality.

The hamiltonian, the sum of potential and kinetic energy written as a function of the coordinates and the corresponding momenta, says it all. Pairs of nucleotide bases from the opposing strands of the double helix and linked together by hydrogen bonds are supposed all to be identical. Their mutual interaction is represented by a simple Morse potential in the standard form $D(e^{-ax} - 1)^2$, where D and a are constants and x is the departure of the mutual separation from the potential energy minimum, taken to be zero. The value of the Morse potential is chiefly that a Schrödinger equation with such a potential, evidently more realistic than

the symmetrical potential energy derived from a linear force law, can be solved exactly (if with some difficulty).

That takes account of interactions within base pairs. Peyrard and Bishop choose as coordinates the difference between and the sum of the displacements of each of a pair of bases from their equilibrium positions. If the bases move together in the same direction, the hydrogen bonds are not stretched. Nothing is assumed in the model about the differences between purine and pyrimidine pairs, although the numbers of hydrogen bonds binding them differ.

To allow for the interaction between successive base pairs in a double helix, the model assumes simply that the potential energy includes for each strand of the double helix a term representing the square of the difference between the displacements of successive bases from their equilibrium positions. This must be a great simplification, especially when linked with the other assumption of the model that each base is otherwise free to move as if it were a particle characterized simply by its inertial mass.

Even if DNA molecules were static, one would guess that the phosphate backbone of each strand would provide a more complicated constraint. In reality, since the problem is a dynamic problem in which the backbone will be vibrating as the bases oscillate around their equilibrium positions, one would expect there to be complicated resonant interactions between the different allowable modes.

Yet, in this business, there is more value in a problem than can be solved than in an attempt to specify at the outset all the complications of an ideal solution. The calculation of the partition function and thus of the macroscopic thermodynamic quantities such as free energy is essentially analytical. But the authors have to resort to numerical integration to derive average values of the difference of the displacement of bases in a pair from their equilibrium positions as well as of the square of this quantity.

The outcome is nevertheless satisfactory. For reasonable values of the constants in the Morse potential energy and for the harmonic interaction between the bases of successive pairs, the average difference of displacement does indeed rapidly increase with increasing temperature over a narrow range of temperature. Using plausible values for the hydrogen

bond interactions between bases within a pair, Peyrard and Bishop work backwards to derive a value for the force constants linking the displacements of bases in successive pairs from the known increase of ultraviolet absorption with increasing temperature.

This model is not as far divorced from previous attempts at the problem of DNA as its form might suggest. Indeed, their hamiltonian can be reduced to equations of motion for what is, after all, a kind of one-dimensional lattice which can then be made to yield nonlinear equations of motion known to allow of soliton solutions. Indeed, the authors raise the intriguing possibility that the onset of denaturation may represent a concentration of vibrational energy at particular points. Would it not be fun if it turned out that these places are those now recognized in molecular genetics as upstream regulatory sequences, TATA boxes and the like?

But what are the chances that it will ever be possible to tackle problems that realistic? Nobody can tell without trying. Yet there are many obvious ways in which Peyrard and Bishop's model of DNA might be further elaborated without running into a computational mess. Having carried the argument so far, it is surprising that they have not already taken it just a little further to calculate the correlations of fluctuations of neighbouring or nearby base pairs. Grafting onto the model a realistic version of the phosphate backbone is another obvious task.

The authors themselves say that they can see ways in which real-life inhomogeneities arising from the presence of four possible bases (A, T, C and G) on each strand might be handled: at one crucial stage in their calculation, it is necessary to determine vibrational wavefunctions at one base from the corresponding wavefunction at the previous base pair, so that it should not in principle be impossible to do that in four (or sixteen) different ways.

Whether people will apply themselves to these problems is another matter, but there are two hopeful signs. What Peyrard and Bishop have done is genuinely interesting in its own right. And while what they have done does not directly compare with climbing Everest, so that the adage that it is easier to climb Everest a second time does not apply, at least they have done the equivalent of climbing Mount Hamilton, which must also make an attack on Everest easier.

John Maddox

Mutating and gating

Richard W. Aldrich

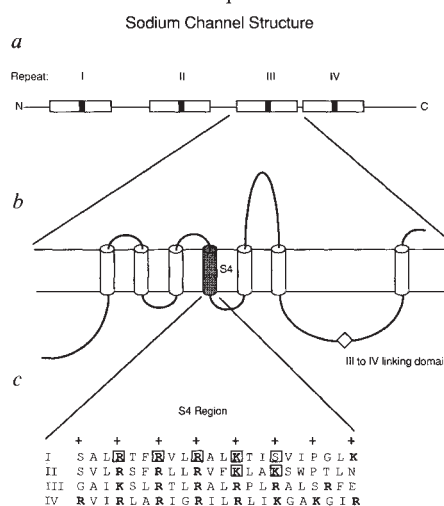
VOLTAGE-dependent gating of ion channels underlies various cellular functions, from transmission of information in the nervous system to excitation of skeletal and cardiac muscle and secretory cells. The mechanisms for opening and closing these channels involve controlling molecular conformation by the voltage across a cellular membrane, much as conformational changes in other proteins are influenced by, for example, ligand binding or photon absorption. The paper by Stühmer, Numa and collaborators on page 597 of this issue¹ demonstrates the first mutational analysis of the mechanisms of voltage-driven conformational changes in sodium channels from rat brain. The report sets the scene for detailed analysis of the mechanisms of voltage-dependent gating through a combination of *in vitro* mutagenesis and electrophysiological experiments on the altered channels in heterologous expression systems, in this case the popular *Xenopus* oocyte.

The essential theoretical framework for voltage-dependent gating comes from the work of Hodgkin and Huxley² in the late 1940s and early 1950s. These authors studied the voltage-dependent sodium and potassium channels in squid axons at a time when the molecular nature of the membrane permeability changes was entirely unknown. Despite this, they astutely predicted several important features of the channel molecules that are only now being confirmed. They supposed "that sodium movement depends on the distribution of charged particles . . . which allow sodium [ions] to pass through the membrane when they occupy particular sites in the membrane" and that the steepness of the relationship between the probability of a channel being open and membrane voltage would depend on the number of charges that move across the membrane for the channel to open. Hodgkin and Huxley believed that "details of the mechanism will probably not be settled for some time, but it seems difficult to escape the conclusion that the changes in ionic permeability depend on the movement of some component of the membrane which behaves as though it had a large charge or dipole moment".

Because the opening of sodium channels after a step change in voltage is transient, Hodgkin and Huxley characterized two independent processes: activation and inactivation, both of which were individually voltage-dependent and therefore required movement of charge through the membrane. These moving charges would create a small outward 'gating' current preceding and coinciding with opening

and closing of the channel.

When the predicted gating currents were eventually measured in the 1970s they were found to be inconsistent with some of the predictions of the Hodgkin and Huxley model³. The most important findings from gating-current experiments were the lack of intrinsic charge movement associated with inactivation, and that inactivation seemed to work by immobilizing the activation charge, thereby contradicting the Hodgkin and Huxley idea of independence of activation and inactivation. Experiments done with



Structure of the sodium channel. **a**, The sodium-channel α -subunit consists of a string of four internally homologous repeats, indicated by the boxes labelled I–IV, linked together by cytoplasmic linkages. The black bars in each box indicate the S4 regions. **b**, Each of the repeats has a proposed structure consisting of six membrane-spanning regions, shown here for repeat III. The S4 region, thought to act as a voltage sensor, is shaded. **c**, The amino-acid sequences of the four S4 regions. They have a conserved structural motif of basic amino acids (arginine or lysine, R or K), indicated in bold. The amino acids that have been mutated by Stühmer and collaborators¹ are enclosed by the squares. The mutations in the III-to-IV linking domain are indicated by a diamond.

single sodium channels have further supported the coupling of activation and inactivation⁴.

Another key result made at about the same time was the discovery that internally applied proteolytic enzymes could abolish the ability of sodium channels to inactivate⁵. The lack of charge movement across the membrane associated with inactivation, and the ability to modify it by cytoplasmic proteases, led Armstrong and Bezanilla to propose a 'ball-and-chain' model for inactivation, where the inactivating conformational change involves the binding of a cytoplasmic domain of the

channel protein to the pore-forming part of the protein, plugging the pore.

These results set the stage for the current molecular advances. The early physiological work allowed predictions about the type of molecular changes underlying gating. In particular, the existence of charged residues lying in the membrane and moving to couple membrane voltage to the opening conformational change, and a cytoplasmic region that would act as an inactivation gate. When the first amino-acid sequence of a sodium channel was reported by Numa's group in 1984⁶, there was considerable excitement and speculation about the domains of the protein which were candidates for the physiologically predicted activation charge and inactivation 'ball'.

Numa and collaborators pointed out a very interesting region of the sequence, S4, that is about the right length to cross the membrane and contains positively-charged amino acids at every third position, with intervening hydrophobic residues. Because the sodium-channel sequence contains four internally homologous domains, four of these S4 regions are present in the channel. The authors suggested that "each of the four positively charged segments . . . acts as voltage sensor, thus being involved in the activation gate". As sequences for other voltage-gated channels were reported it became clear that S4 is very highly conserved in this class of proteins, further supporting the idea that it is of central importance in voltage-dependent gating^{7,8}.

Stühmer *et al.* in their paper in this issue¹ report the next step — altering the positively charged residues in S4 and determining the effect on gating. If the S4 charges represent the actual gating charge, then neutralizing them (by replacing the arginines or lysines with glutamine) or reversing their sign (by changing them to glutamic acid) should reduce the steepness of the relationship between the probability of a channel opening and voltage. Neutralizing or reversing the sign of the positive amino acids in S4 of the first homologous repeat indeed reduces the steepness of the voltage dependence, roughly proportional to the amount of positive charge changed. This result provides the first evidence that the S4 charges actually represent the gating charge for channel opening. Curiously, neutralizations of one or two positive charges on S4 of the second homologous repeat do not significantly affect the voltage dependence of gating. The authors find this result difficult to interpret as they have not systematically investigated the second homology repeat. Taken at face value, however, this at least implies that the gating machinery is somewhat more complicated than the four individual S4 helices moving independently through the membrane's electric

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field, causing a channel to open.

Stühmer *et al.* also identify a potential functional domain of the channel protein as a likely candidate for at least part of the inactivation 'ball'. According to commonly accepted views of the folding pattern of the channel protein through the membrane, the four repeats are linked by cytoplasmic domains (see figure). The shortest of these, between the third and fourth repeat, must be intact for normal inactivation. Oocytes injected with messenger RNA encoding only repeats I, II and III do not produce functional channels. When this messenger RNA is co-injected with that encoding repeat IV, channels are expressed that do not inactivate. The resulting whole-cell and single-channel currents are very similar to those of intact channels treated with internal trypsin, suggesting an identification of the cytoplasmic linking domain between repeats III and IV with the site of proteolytic cleavage. In fact, this domain contains a high density of potential trypsin-cleavage sites. A similar experiment cutting between repeats II and III produces channels with intact inactivation. Additional evidence supporting the view that the III to IV cytoplasmic-linker domain is involved in inactivation comes from experiments showing that antibodies directed to this sequence can slow the inactivation of native channels⁹.

The importance of the present work is that it grounds the inferences about channel structure made from electrophysiological experiments in concrete molecular structure, and signals the beginning of a new and exciting phase in the investigation of channel gating. In retrospect, it is evident that each advance in our understanding of voltage-dependent channel gating has risen from the development of new techniques for measurement and manipulation of channels, such as voltage-clamp, gating currents, specific pharmacological agents and single-channel recording. The latest, and probably most powerful, breakthrough of site-directed mutagenesis combined with electrophysiological study of function promises much. And if the consequences of past breakthroughs can be taken as an indication, we will probably learn much that is quite unexpected. □

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Making light of gravity

Ethan Vishniac

THE most critical problem in cosmology today is the task of explaining the existence of galaxies and galaxy clusters. This problem is particularly acute because the only real success of modern cosmology is the use of a perfectly homogeneous and isotropic model to explain the primordial abundances of the lightest elements and the existence of the microwave background. Most research in this field has been concerned with understanding how gravitational instabilities can take small perturbations in such a Universe and build regions of large density contrast. However, no totally satisfactory model has resulted, largely because the gravitational growth of structure is relatively slow in an expanding Universe. Recently, Craig Hogan and Simon White argued¹ for the revival of an alternative, "mock gravity", in which radiation is invoked to provide an attractive, inverse-square-law force. The basic idea is not particularly new: the first paper² on the topic was published in 1941 and the first application to galaxy formation, due to George Gamow³, appeared in 1949. In his most recent paper⁴, however, Hogan argues that the recent detection of an anomalous component to the cosmic microwave background⁵, predicted by Hogan and White in their earlier paper, provides fresh evidence for the existence of a background radiation field of sufficient intensity to drive clustering in the early Universe.

The basic idea of mock gravity is quite simple. An isotropic radiation field filling the Universe will provide an isotropic pressure. Two adjacent clouds of gas can cast shadows on each other, thereby creating a net force on each cloud which will tend to drive them together. The angle subtended by a cloud, and therefore the strength of the effect, will diminish as the inverse square of the distance to the cloud. Therefore the apparent force exerted by the cloud will be universally attractive and obey an inverse-square law, just like gravity. Unlike gravity, the strength of the force is not given by a Universal constant, but is instead a function of the intensity of the radiation field and the opacity of the clouds. For a sufficiently intense radiation field the strength of the mock gravitational forces can be much greater than the strength of normal gravitational interactions. George Gamow suggested that the microwave background radiation (at that time unobserved) would tend to squeeze together the electrons (and therefore the baryons) in the primordial plasma. This idea suffers from the fatal difficulty that the electrons and the photons in the primordial plasma were in nearly perfect thermal equilibrium.

As a result, a cloud of matter in the early Universe will emit and scatter compensating amounts of radiation into paths that would otherwise be depleted by the cloud. This flaw does not appear in Hogan and White's theory because the driving radiation field is produced by an early generation of stars, and consequently has a characteristic temperature of thousands, or tens of thousands, of degrees. The dust responsible for absorbing and scattering this radiation would be heated to temperatures of only tens (or at most hundreds) of degrees kelvin.

For Hogan and White's proposal to work, the Universe needs to produce a large quantity of dust and radiation when its age is some tens of millions of years. The dust would be the natural result of the formation of an early generation of massive stars. The energy requirements are somewhat more severe, and it may be that the energy supplied by ordinary nuclear burning is insufficient. This is not an insurmountable obstacle because gravitational accretion onto very massive objects in the range of hundreds of solar masses would produce energy with the required efficiency. The origin of these objects is neither more, nor less, mysterious than the origin of an early generation of stars and does not form a part of this theory. Even if the dust production is modest, Hogan suggests that under these conditions the intergalactic medium may naturally clump into small clouds whose atomic opacity is sufficient to produce mock gravitational effects.

One of the most frequent criticisms levelled at cosmological theories is that they are devoid of testable predictions. Fortunately, mock gravity yields at least three. First, the fact that large-scale structure is due not to gravitational forces acting at the present, but to forces acting at some time in the past, implies that the observed amount of clustering in the Universe will not be a strong function of redshift. This prediction is at least consistent with current observations and is undoubtedly testable.

Second, the radiation field that drives mock gravity may or may not be directly observable, but the radiation absorbed by the dust (or gas) will be re-emitted at wavelengths slightly above the cosmic microwave background peak. This is the prediction of Hogan and White which has been vindicated by the observations of Matsumoto *et al.*⁶ (discussed in detail by Carr in News and Views last year⁷).

Third, this radiation will be significantly more anisotropic than the cosmic microwave background itself. It follows that temperature anisotropy measurements of

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the microwave background at short wavelengths should find a signal as high as 10^{-3} . Current limits at long wavelengths are down to 10^{-5} .

Hogan and White's proposal has come under fire in a new paper by Wang and Field⁷. They recalculate the growth-rate including a number of effects neglected by Hogan and White. The authors conclude that inclusion of the radiation drag caused by the microwave background, as well as the limited coupling between the dust and gas, causes mock gravity to fail to produce significant growth of structure on all scales of cosmological interest. This result is not, by itself, fatal. Wang and Field have not explored the full range of parameter space, nor have they considered the role suggested by Hogan for atomic opacities. It seems clear that the struggle to establish mock gravity as a plausible model for galaxy formation is not over, and that a number of important quantitative issues will have to be resolved favourably for it to gain respect among cosmologists.

As data accumulate on the motions of galaxies and their large-scale distribution, theories of galaxy formation have shown a disturbing tendency to evaporate, leaving the community of cosmologists groping

for a satisfactory model. It may be that the reappearance of mock gravity is more a sign of quiet desperation than of a breakthrough. Still, the failure of simple gravitational-instability models to satisfy the available constraints would seem to be a strong hint that a more radical approach is necessary. Moreover, the existence of a substantial component to the cosmic microwave background that does not originate in the very early Universe is, if confirmed, proof that our current understanding of the evolution of the Universe from recombination has some gaping holes. As a hypothesis, mock gravity has the distinct advantage that it uses plausible physics and makes definite predictions. Whether it can be transformed into a workable theory of the origin of galaxies is an extremely uncertain question. □

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SITE-DIRECTED MUTAGENESIS

Reversing enzyme specificity

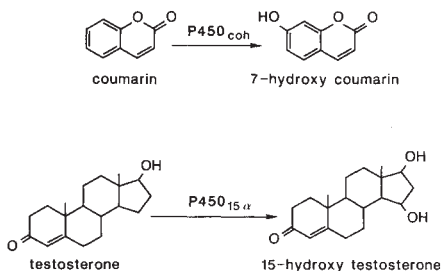
Thomas L. Poulos

ON page 632 of this issue¹, Lindberg and Negishi describe how a single amino-acid substitution can completely reverse the specificity of an enzyme. The enzyme in question is cytochrome P450. P450s, haem-containing proteins, catalyse the O_2 /NADPH-dependent hydroxylation of aromatic or aliphatic molecules. Liver microsomal P450s rid the system of toxic hydrocarbons by hydroxylating these molecules, rendering them more soluble for elimination from the body. These P450s tend to have rather broad specificities as required by their function to handle different organic molecules. By contrast, mitochondrial P450s exhibit much higher levels of specificity as they participate in the production of specific steroids.

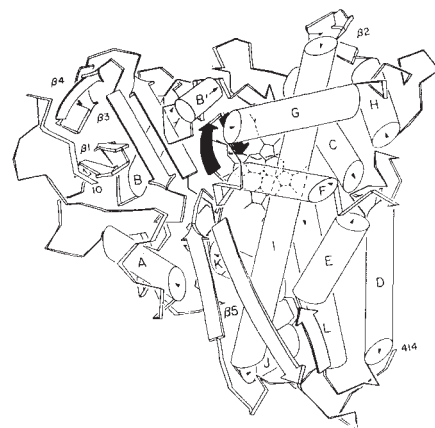
Interest in P450s ranges from how they metabolize drugs and other xenobiotic compounds to how specific P450 genes are regulated. As well as the details of the actual hydroxylation reaction, the main enzymological question centres on substrate specificity. The basic hydroxylation chemistry will probably turn out to be the same for all P450s, given that all these enzymes have exactly the same haem group and conserved cysteine haem ligand. Yet there must be significant differences in the substrate-binding pocket, given the many different P450 substrates. Understanding the structural

basis for these differences is one of the central goals in P450 research.

Recent advances in cloning and expression of P450 genes² have opened the way for the use of mutagenesis techniques to address the relationship between amino-acid sequence and substrate specificity. Lindberg and Negishi¹ have now developed such expression systems for two P450s called P450_{coh} and P450_{15α} which catalyse the reactions shown below.



What makes these two P450s so interesting is that they differ in only 11 out of 494 amino acids, yet coumarin is 170 times better as a substrate for P450_{coh} than is testosterone, whereas P450_{15α} does not hydroxylate coumarin. Lindberg and Negishi changed each of the key 11 amino acids in P450_{coh} to the corresponding residue in P450_{15α} to try to identify which of them is or are responsible for substrate specificity. The most important change



Schematic representation of the P450_{cam} crystal structure taken from ref. 4. Helices are labeled A–L. The substrate (not shown) would be positioned over the haem between the central region of helix I and the inner strand of β 3 and just below the carboxy-terminal end of helix F. The suspected substrate access channel lies between the loops connecting helices F and G and the β -strands. The shaded β -pair represents the conserved peptide containing the cysteine haem ligand.

is at position 209 where P450_{coh} has a phenylalanine and P450_{15α} a leucine. If Phe 209 is replaced by Leu in P450_{coh}, testosterone is now a slightly better, rather than a considerably worse, substrate than coumarin. The reverse experiment is nearly as dramatic. That is, replacing Leu 209 with Phe in P450_{15α} enables the mutant P450_{15α} to operate on coumarin where wild-type P450_{15α} has no activity against coumarin. Moreover, the Leu 209-to-Phe mutant in P450_{15α} is about 125-fold less active towards testosterone than is the wild-type enzyme. This complete reversal of specificity resulting from a single amino-acid substitution is a remarkable finding considering that the two substrates are very different in size and shape, although they do share a common structural feature, a keto-oxygen atom.

The central question, of course, is the structural basis for this change in specificity. Unfortunately, a critical piece in the puzzle is missing: the crystal structure of a mammalian P450. Although more than 60 P450 sequences are known³, the crystal structure of only one P450, P450_{cam} from *Pseudomonas putida*, has been determined⁴. The bacterial and mammalian sequences are very similar in regions critical to haem binding, but the much larger mammalian P450s (about 500 residues versus 414 for P450_{cam}) make precise modelling of mammalian P450s using the bacterial X-ray structure a risky business — especially when attempting to pinpoint specific residues. Nevertheless, exhaustive sequence alignments and secondary structure predictions⁵ suggest that the basic helical motif and architecture shown in the figure is conserved in the mammalian P450s. Using secondary structure predictions for mammalian P450s

and the P450_{cam} X-ray structure as guides, position 209 in P450_{coh} and P450_{15a} should occur in helix E (see figure).

This raises an interesting problem. The dramatic effect of changing residue 209 suggests a critical role in substrate binding, such as direct contact with the substrate or perhaps a structural role in defining a substrate access channel. However, helix E is too far away from the haem substrate pocket and the access channel to serve either function. This means either that residue 209 has some indirect role in substrate binding, which implies long-range structural effects due to a single amino-acid substitution, or that the various predictions for mammalian P450 structures are not very close to the truth. Although the latter possibility is

rather disconcerting, the database simply is too limited to draw any firm conclusions one way or the other. Nevertheless, Lindberg and Negishi's provocative results should provide food for thought, and similar mutagenesis studies should yield valuable insights into the structure of P450 active sites in the near future. □

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SOLID-STATE PHYSICS

Back to basics for microchips

J.B. Pendry

IN the most recent of a spate of micro-electronic studies made possible by improvements in semiconductor fabrication techniques, M. Henini *et al.* (*J. Phys. Condens. Matter* **1**, 3025–3030; 1989), report experiments in which a voltage is applied across a double-barrier device: the electrons cross a narrow, high barrier, pass through a region of constant potential and finally emerge through a second narrow barrier (Fig. 1). Far from obeying Ohm's law, showing a simple linear variation, the current is highly structured. The first derivative (the 'resistance' of the device) shows marked oscillations with voltage which in some instances even have negative excursions. What is going on? The device has finally learnt basic quantum mechanics and is showing resonant

transmission through a barrier when the wavelength matches the barrier width.

Many of us are familiar with the basic concepts of quantum mechanics — idealized one-dimensional systems, particles in boxes and so on — which make quantum mechanics seem like a branch of optics. But from real semiconductor devices we soon learn that electrons really behave like classical particles with a few quantum corrections to the mass. Newton was right after all. The problem is that in conventional devices, electrons travel large distances through rather impure material. They bounce off lattice vibrations and other imperfections all of which conspire to randomize the phase of each electron's wavefunction. It is, of course, the phase which distinguishes a quantum

from a classical particle.

All this has changed in a series of papers appearing over the past year. That by Henini *et al.* is a particularly new and striking example. Two key technical developments lie behind their remarkable result. First, the devices are physically very small. The barriers can be as narrow as 0.25 nanometres (but in this instance were of the order of 100 nanometres). Second, and of greater importance, the gallium arsenide out of which the device is made is prepared in an unusually pure and perfect state by the technique of molecular-beam epitaxy. As a result electrons can cross the barrier ballistically without losing any of their phase coherence by scattering from imperfections: the experiment regains ideal classroom simplicity.

Chemists and biologists will be familiar with a related set of oscillations which occur in the X-ray adsorption cross-sections of solids. Here an X-ray kicks an electron out of the inner core of an atom, but the escape of this electron is impeded by the surrounding shells of atoms which behave like cages. Matching the electron wavelength to the cage radii produces oscillatory structure as the electron energy is tuned by increasing the X-ray energy. This is a valuable technique for measuring radii of coordinating shells of atoms, but differs from the experiments reported here in the much higher electron energies involved: hundreds rather than one or two electron volts.

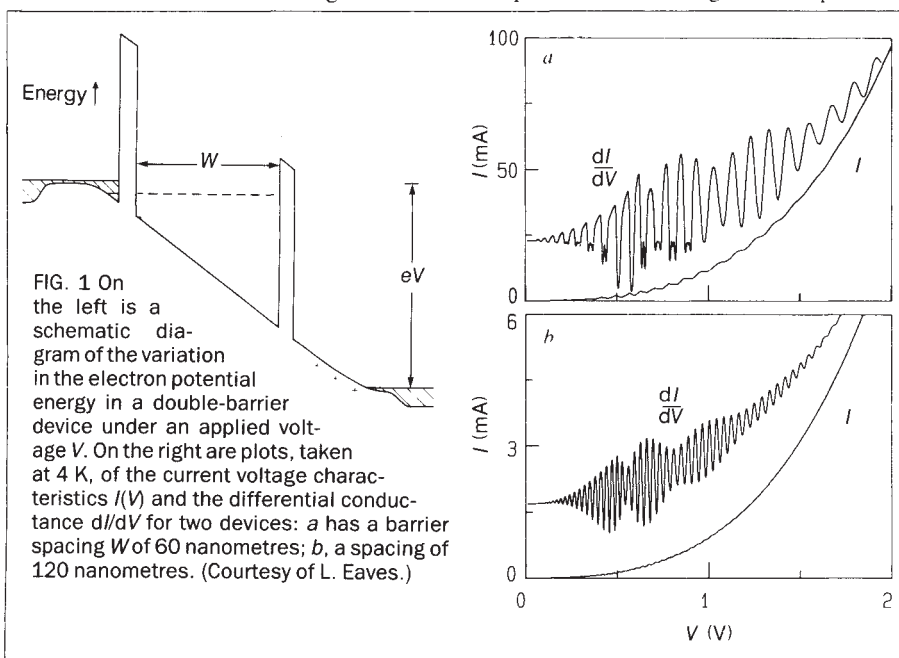
The modulations of intensity shown in Fig. 1 contain a wealth of structure. The most striking is the rapid oscillation of the differential conductance, $G = dI/dV$. Waves penetrating the left-hand barrier rattle around in the gap (width W) between the two barriers: not all of them escape from the right hand barrier at the first attempt, some reflect but return again after bouncing back from the left barrier. That is where quantum mechanics enters because the transmission is enhanced if the waves interfere constructively.

They do so if their phase changes by an integral multiple n of 2π on making the round trip between barriers: $k \times 2W = 2n\pi$, where k is the wavevector of the electrons which depends on the applied voltage through the energy, E , of the electrons. Hence the voltage spacing between successive peaks in G is given by

$$\delta V = (\pi/eW) dE/dk = \pi\hbar/(e\tau)$$

where $\hbar^{-1}dE/dk$ is the group velocity of the electrons and τ is the time taken by the electron to make a return trip; e is the electron charge and $\hbar$, Planck's constant. The device is a sort of stop watch which times the electrons over a measured distance.

One of the beauties of this experiment is the structure within structure which it exhibits. Viewed carefully, the oscillations of dI/dV in Fig. 1 show modulations



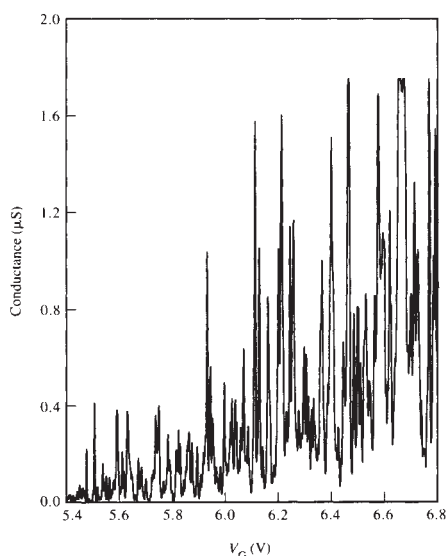


FIG. 2 Conductance of a silicon-metal-oxide field-effect transistor as a function of gate voltage measured at 50 mK. Note the intense fluctuations which are reproducible as a function of time. (From A.B. Fowler, J.J. Wainer & R.A. Webb, *IBM J. Res. Devel.* **32**, 372–383; 1988.)

in their amplitude. These are particularly pronounced in Fig. 1b. At voltages above about 0.5 V the electrons crossing the right-hand barrier have energies greater than the barrier height and this provokes a second set of resonances within the much narrower barrier defining the left-hand edge of the well. From the equation for δV one can see that a smaller value of W results in more widely spaced oscillations, and hence the slow modulation of the current.

In fact observation of this effect illustrates yet another feature of quantum mechanics. Classically we expect that if an aeroplane flies above the height of a mountain it will pass safely over the top; otherwise it will crash. Quantum mechanics disagrees, predicting that even if a particle has enough energy to pass over an obstacle, it may still have a chance of being reflected. Here is the evidence that this does happen.

Quantum mechanics produces surprising, non-ohmic, features in the conductance of small samples. *In extremis*, the differential conductance dI/dV can be negative. The presence of negative resistance in a circuit almost always leads to instabilities of which there is evidence in Fig. 1a: the minima in dI/dV show a curious clipped structure, evidence that the circuit is breaking into spontaneous oscillation. Such instability can be exploited in microwave generators and fast switching diodes as reported in a different context by Serge Luryi in News and Views (*Nature* **336**, 515–516; 1988).

Extreme fluctuations of conductance are not confined to devices of the highest perfection, though it is true that they can be most effectively exploited in this case. In a strongly disordered semiconductor,

random variation of the potential forms barriers and wells which trap electrons. In this way strong disorder acts mainly to reduce the conductance to a very low value but, if electrons injected into the disordered material are degenerate with one of the trap energies, very large transmission can be seen over an extremely narrow voltage range. In thicker samples an electron can be trapped for a time in one region of the solid, receive a small amount of energy from lattice vibrations and jump into another trap with nearly the same energy. A chain of nearly degenerate traps stretching across the solid would provide a conducting path for electrons of the appropriate energy. Change the voltage a little, the electrons are no longer degenerate with the traps, and the path is switched off. Conductance-voltage plots for strongly disordered materials characteristically show a very spiky conductance

which resembles noise, except that it is reproducible when the voltage is recycled (Fig. 2).

Provoking to theorists as these properties of strongly disordered semiconductors may be, they are uncontrolled and little understood. Exploitation must depend on the well understood quantum effects now being observed in very perfect devices. The importance of these new experiments is threefold. Experimentalists can now control small semiconductor devices to an unprecedented degree. This means that experiments can be simple enough in theoretical terms to explore some important and fundamental problems in physics. At the same time we can expect a valuable spin-off in terms of practical applications. □

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PALAEOMAGNETISM

Fast changes in geomagnetism

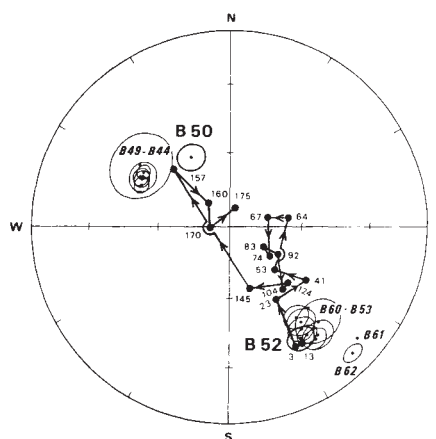
Mike Fuller

A SEQUENCE of palaeomagnetic directions corresponding to the “astonishingly high rates of change . . . of at least 3° and 300 gammas [nanotesla] per day” have been identified from lava flows in Oregon by Robert S. Coe and Michel Prévot (*Earth planet. Sci. Lett.* **92**, 292–298; 1989). Typical rates of change currently are a few degrees per century, with the changes of local field intensity attributable to internal sources being of the order of hundreds of gammas over years or decades. During a reversal, the decay of the main dipole field of the Earth enhances the local-field changes, but the rates interpreted by Coe and Prévot remain astonishing.

The morphology of the transitional fields during geomagnetic reversals has become a principal concern for palaeomagnetists. The hope is that a detailed knowledge of the transitional fields will reveal some key aspect of the geodynamo. Records of the sequences of field changes during a reversal have been obtained at several sites. The Steen's Mountain reversal record (dating from 15.5 ± 0.3 million years ago), used by Coe and Prévot, is one of the most complete. Coming from an immense pile of Miocene lava flows on the California/Oregon border, the record was first studied by the late Norm Watkins in the 1960s. More recently, it has been reinvestigated by a group from the US Geological Survey, the University of California at Santa Cruz and the Université des Sciences et Techniques at Montpellier. Coe and Prévot, members of this group, made their new discovery in a detailed extension of this study.

The field changes they identify, if real,

are very important as a new aspect of field behaviour during reversals. Moreover, the fact that such rapid magnetic field fluctuations were not electromagnetically screened by the mantle would require a downward revision of our estimates of the electrical conductivity of the lower mantle. The authors are suitably cautious about uncritical acceptance of the result noting that the “rapidity and large amplitude of geomagnetic variation . . . even when regarded as an impulse during a polarity transition, truly strains the imagi-



The directions of magnetization of the flow B51 obtained after demagnetization to 500 °C. They are interpreted to be recording rapid changes in the geomagnetic field during the Miocene Steen's Mountain reversal. The flows are numbered from bottom to top of the section, so that the flow number increases with age. The directions in flow B51 are assigned numbers indicating their height above the base of the flow in centimetres. The directions in B51 appear to fill in the gap between flows B50 and B52. (From Coe and Prévot.)

nation...". The critical question is whether the directions of magnetization observed are true records of field changes.

Palaeomagnetism has a history of giving shocks to the geological and geophysical community. Usually these are initially unpalatable, although they are later accepted. Such shocks include the magnetic evidence for continental drift, sea-floor spreading, block rotations within continents and geomagnetic reversals themselves. Are the changes interpreted by Coe and Prévot another shock, with which we will learn to live, or are we being fooled by the magnetism of the rocks?

The observation seems to be straightforward. Measurement techniques were standard. The results come from lavas, whose magnetization is in general amongst the easiest to interpret. The magnetization is acquired as the rock cools through the blocking temperatures of its magnetic phases. Treating magnetization as a typical relaxation phenomenon, L. Néel showed (*Adv. Phys.* **4**, 191–243; 1955) that on cooling through this blocking temperature, the relaxation time of the magnetization of a fine particle increases spectacularly. The magnetization ceases to be in thermal equilibrium with the ambient field and becomes stably blocked, so that it can survive over the geological aeons. The field in which the flow cools determines an energetically preferred direction and so is recorded as a statistical bias in the magnetization blocked in the sample. This we later measure at room temperature, as the natural remanent magnetization.

The critical lava in the new study (see figure) is B51. The nine flows immediately below it record similar directions, represented in the figure by flow B52. The flow immediately above, B50, records another direction. The six flows above it record statistically indistinguishable directions, which differ only slightly from B50. The directional change between B50 and B52 is almost 90°. After thermal demagnetization to approximately 500 °C, the directions in flow B51 fill in the gap in the record systematically, with the lower samples having directions similar to the lava below and the upper samples having directions similar to B50. Individual samples from near the base of the flow have magnetizations similar to those in the lava above blocked below 300 °C, but directions similar to those in the lava below blocked in the highest temperature range.

Using a standard conduction cooling model, the authors calculate the time taken for the whole of flow B51 to cool to 500 °C to be 15 days. Assuming that the flow cooled as a single flow, this estimate is not likely to be in substantial error. An order-of-magnitude estimate of the cooling time, based on a dimensional analysis of the diffusion equation, shows that the



100 years ago *Scientific worthies*

DMITRI Ivanowitsch Mendeleeff was born on 7 February 1834, at Tobolsk, in Siberia. Having passed through the Gymnasium at Tobolsk, Mendeleeff, at the age of sixteen, was sent to St. Petersburg, with the intention that he should study chemistry at the University, under Zinin. In 1856 he was admitted to the degree of *Magister Chemiae* of the Physico-mathematical Faculty of the University of St. Petersburg, and was made *Privat-Dozent* in the University.

In 1859, Mendeleeff repaired to Heidelberg, where he established a small private laboratory, and occupied himself with the determination of the physical constants of chemical compounds. He returned home in 1861, and in 1866 he became Professor of Chemistry at the University. He is now Emeritus Professor, and delivers annually a course of lectures on general chemistry.

Mendeleeff is so prolific a writer that it is impossible within the limits of an article of this kind to do justice to his work. There is, in fact, no section of chemical science which he has not enriched by his contributions. But his reputation mainly rests upon his contributions to



physical chemistry and to chemical philosophy.

No man in Russia has exercised a greater or more lasting influence on the development of physical science than Mendeleeff.

From *Nature* **40**, 193; 27 June 1889.

estimate cannot be in error sufficiently to change the basic conclusion of the authors. Hence, the implied field changes are indeed astonishingly rapid.

Faced with this situation, the authors attempt to explain the observations in terms of complications in the recording process. They investigate various possibilities, such as resetting of the magnetization by heating by the flow above and contamination by viscous remanent magnetization. Baking by the overlying flow accounts for some of the observations, but not all. One would not expect the complications in the path seen in the figure if the explanation were simple reheating. Being unable to account convincingly for the observations with such explanations, they conclude that the observed magnetization most probably recorded rapid field changes. However, an additional serious difficulty with this interpretation is that not only must very rapid field changes have taken place, but the change in direction between B52 and B50 must have happened in just the 15 days in which B51 cooled. This seems so unlikely that one is encouraged to return to explanations based upon some unrecognized complexity in the recording process.

There are further puzzles in the possible effects of reheating and resetting of magnetization in flow B51 by B50. If the swing in high-temperature magnetization direction in the top metre of B50 is due to reheating, as suggested by the authors, the flow must have been heated to more than 500 °C to a depth of 1 m. This suggests that the heat transferred was more than that to be expected from the cooling of a single 2-m flow. Perhaps fluids were circulating in B51; or perhaps a cooling unit larger than the single flow was responsible

for reheating B51. No matter what the cause of the reheating of B51 may have been, some special mechanism must have been active to explain reheating to 500 °C at 1 m into the flow. However, once one accepts reheating as a mechanism explaining observations at a depth of 1 m into a 2-m flow, it seems plausible to use the same explanation for the whole phenomenon. The price one must pay is to say that the complication in the path illustrated in the figure is an unexplained perturbation of the pattern of magnetization.

Coe and Prévot describe their result in detail and give their interpretation with suitable caution. Whether their explanation is correct, or whether some sleight of hand of the recording process has misled us, remains to be seen. Because it is a well-documented result, we can all puzzle over it. At the risk of being excessively conservative, I would ask for some further demonstration that the result cannot be explained by reheating of the flow, before accepting that rapid field changes are recorded. This is in keeping with a personal impression that to obtain reversal records and to demonstrate their fidelity is at the very limits of palaeomagnetism. Most would probably agree that the time taken for the field to reverse is of the order of thousands of years, that the field intensity decreases by about an order of magnitude and that the field direction and intensity fluctuate more rapidly during reversals than usually. However, there is little agreement on further details, so that we are still a long way from having a proper description of transitional fields. □

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The gene and its product

J.L. Mandel

SEVERAL recent articles reflect the rapid pace of research on the dystrophin gene, whose malfunction is responsible for the Duchenne and Becker muscular dystrophies. The most recent example is the report by Love *et al.*¹ that the dystrophin gene has a close relative on human chromosome 6. This finding stimulated particular interest at a recent meeting*, as it may provide an explanation for the poorly understood but striking differences in phenotypic expression elicited by the absence of dystrophin in man and mouse, or account for one of the autosomal muscular dystrophies.

The dystrophin gene is now well known to be a "mammoth" gene (as coined by V. McKusick). The most recent pulsed-field gel electrophoresis data by G. J. Van Ommen (University of Leiden) suggest it is 2.3 megabases in size — 12 times the size of the coagulation factor VIII gene, one of the largest genes known previously, and 1,500 times that of the β -globin gene. A similar size range is observed in dystrophin from mouse or chicken (L. M. Kunkel, Children's Hospital, Boston; R. Heilig, University of Strasbourg). The number of exons is not yet known exactly, but is more than 75. The protein sequence deduced from analysis of the human complementary DNA by Koenig *et al.*² suggested a multidomain structure (see Fig. 1). The 240-residue amino terminus has strong similarity to the amino-terminal actin-binding domain of α -actinins (a family of cytoskeletal and myofibrillar proteins). The largest part of the protein comprises 24 repeats of a roughly 109 amino-acid-long unit (with rather loose homology between them) that shows weak similarity with similar sized repeats found in α - and β -spectrins and in α -actinin. This spectrin-like domain is assumed to have an extended rod shape and is interrupted by two proline-rich tracks found after repeats 3 and 19, hypothesized by M. Koenig (Children's Hospital, Boston) to act as hinge regions. A third domain (150 amino acids) is cysteine-rich and is similar in sequence to the carboxy-terminal domain of α -actinin from slime mould that includes two calcium-binding sites (the corresponding sites may not be functional in dystrophin). The origin of the carboxy-terminal domain (420 amino acids) is more mysterious as it has no similarity to other known protein sequences. The last two domains are by far the most conserved in evolution, as shown by comparison of the human and chicken cDNA¹.

By serendipity, K. Davies group (John

Radcliffe Hospital, Oxford) has now isolated a human muscle cDNA clone that is very similar to the carboxy-terminal domain of dystrophin (83 per cent amino-acid conservation). This cDNA recognizes a muscle messenger RNA of 13 kilobases, a size very similar to that of dystrophin mRNA¹. Extension of the cloning should reveal whether the homology extends to the spectrin-like and α -actinin-like domains, and whether this new autosomal gene encodes a ' β -dystrophin' or whether the homology is more partial and the result of exon shuffling.

Spectrin and α -actinin both form antiparallel dimers, a homodimer in the case of α -actinin and a heterodimer composed of α and β subunits in the case of spectrin. Thus dystrophin is assumed (without experimental evidence yet) to have a similar structure: a homodimer seems more

scopy studies suggest a lattice organization (S. Watkins, Dana Farber Cancer Institute, Boston³) and Koenig proposed a model of a flexible hexagonal array (honeycomb) of antiparallel dystrophin dimers. The elegant biochemical studies of K. Campbell (University of Iowa)⁶ show that dystrophin is tightly bound to one of several integral membrane glycoproteins. Identification of the protein(s) that interact with dystrophin in various types of muscle as well as in brain will be an important step towards understanding the function of dystrophin. Some functions have been suggested: stabilization of the plasma membrane; anchoring the cytoskeleton to the membrane; or maintaining a nonrandom distribution of the membrane glycoprotein(s) linked to dystrophin, a function similar to that proposed for ankyrin^{5,6}, itself a spectrin-binding protein.

As well as being expressed in skeletal muscle, the dystrophin gene is also expressed in smooth and cardiac muscle and in brain. An alternative promoter and first

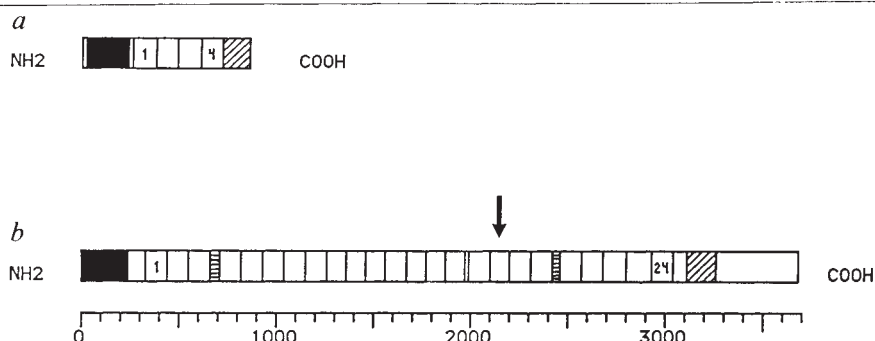


FIG. 1 Domain organization of *a*, α -actinin and *b*, dystrophin. The domains showing similarity between actinin and dystrophin are represented in black (amino-terminal domain) or obliquely hatched (cysteine-rich domain). The 'extended-rod' domain of dystrophin is divided in 24 spectrin-like repeats (the first and last are numbered). Two putative hinge regions are indicated by horizontal hatches. The arrow shows the position of the deletion hot spot (between exons 44 and 45 in the gene). Many Becker deletions extend from this point towards the carboxy-terminus while the putative 'asymptomatic' region proposed by Koenig extends towards the amino terminal. (Figure courtesy of M. Koenig.)

likely than a heterodimer with another dystrophin-like protein, as deletions or duplications in the spectrin-like region that significantly alter the size of the protein can result in a very mild phenotype (see below). In comparison, smaller deletions of repeat units in the gene encoding a collagen chain have catastrophic results in man (dominant osteogenesis imperfecta), because of the heteromeric structure of collagen.

Localization and function

Dystrophin is localized on the cytoplasmic face of the plasma membrane (reviewed in ref. 4; see Fig. 2). It seems to be more concentrated at the myotendinous and neuromuscular junctions and, in particular, large quantities of dystrophin (detected with antibodies against the mouse protein) are found in the electric organ of *Torpedo* (E. Bonilla, Columbia University, New York). Immunoelectron micro-

exon has been found in brain^{7,8} and, curiously, the 5'-untranslated region of brain mRNA appears highly conserved in evolution (99 per cent identity between rat and man), which is not the case for the muscle 5'-untranslated region. The functional significance of expression in brain is further supported by the finding of brain-specific alternative splicing, restricted to the carboxy-terminal domain, that creates isoforms with a different carboxy terminus, as reported by Kunkel⁸. This could allow dystrophin to interact with different proteins in brain than it does in muscle. Alternative splicing could also be responsible for the synthesis of a shorter dystrophin in smooth muscle⁸. The functional significance of dystrophin in brain is still a mystery. It seems to be present at higher concentrations in neurons than in glial cells (but this is not unanimously accepted), without very specific localization to brain structures. The relationship

*Meeting organized by the Muscular Dystrophy Association, Banbury Center, Cold Spring Harbor Laboratory, New York, 2-5 April 1989.

between dystrophin and mental retardation, which is seen in some Duchenne patients, is unclear. Mental retardation does not seem to result from deletions in a specific part of the dystrophin gene and may not segregate consistently within families. The lack of — and the necessity for — well-designed and comprehensive studies on mental retardation in Duchenne patients was stressed by several participants at the meeting.

Deletions and diagnosis

When cDNA probes were first used, it was quickly recognized that deletions in the dystrophin gene are found in about two-thirds of Duchenne patients and of patients with the milder and more slowly progressing Becker form². The size of deletions does not seem to be correlated with severity. This led Monaco *et al.*⁹ to propose that exon deletions that respect the reading frame may lead to the milder form, whereas those that lead to frame-shifts in the mRNA sequence downstream to the deletion would cause the severe Duchenne form.

To assess this hypothesis, *Hind*III genomic DNA fragments that correspond to successive exons were identified and the borders of most exons have now been sequenced (laboratories of Kunkel, R. Worton, T. Caskey and Van Ommen), so that it is now possible in most cases to predict, on the basis of the pattern of deleted genomic *Hind*III fragments detected by a cDNA probe, whether a patient has an 'in-frame' or an 'out-of-frame' deletion.

Exceptions to the frame rule were first reported by Malhotra *et al.*¹⁰ who showed that out-of-frame deletions of exons 3–7, which remove most of the α -actinin-like amino-terminal domain, produce phenotypes that vary from a mild or severe Becker to a Duchenne type, and it was proposed that reinitiation could occur on a methionine codon just downstream to the deletion, giving rise to a truncated protein. Other exceptions were described by Baumbach *et al.*¹¹. In a large multicentre study, 258 independent deletions were analysed and compared with clinical evolution of patients (Koenig). Agreement with the reading-frame model was found in 92 per cent of the cases; 4 per cent of the exceptions were either of the type reported by Malhotra *et al.*¹⁰, or were in-frame deletions of more than 30 exons leading to a severe phenotype. Thus only 4 per cent of the exceptions could not be accounted for¹². Alternative splicing mechanisms such as exon skipping could well provide an explanation for the latter cases. In support of this, J. C. Kaplan (Institut de Pathologie Moléculaire, Paris) reported an analysis of muscle dystrophin mRNA in a deletion patient, using the polymerase chain reaction (PCR), that revealed the presence, in addition to the expected

deleted RNA species, of a minor species which lacks an additional exon adjacent to the deletion.

A puzzling observation made by Koenig is the lack of Becker deletions in the region of exons 31–44 (within the spectrin-like domain), whereas many Becker deletions are found 3' of exon 44 (many deletions have a break point in the intron between exons 44 and 45, see below) and Duchenne deletions are found both on the 5' or 3' side of this intron. This led Koenig to hypothesize that deletions in the region of exons 31–44 that respect the reading frame might result in normal phenotype or in very mild muscular symptoms. A striking confirmation that this may occur was reported by K. Fischbeck (University of Pennsylvania, Philadelphia), who described a large family with X-linked myalgia and cramps but without muscle weakness, and where a relatively large deletion of the dystrophin gene was found

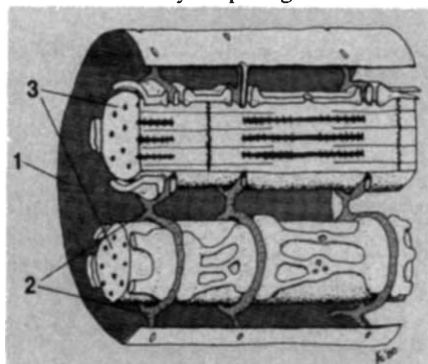


FIG. 2 Schematic picture of muscle cell, sliced down the middle and cut at both ends. Dystrophin is thought to be located inside the surface membrane, or sarcolemma, surrounding the cell (1) and the tunnel-like extensions of the membrane, called T-tubules (2). The T-tubules surround muscle fibrils (3), the contractile elements of the system. (Picture by Abraham B. Eastwood; courtesy of Muscular Dystrophy Association.)

that encompasses exons 10–22 (ref. 13). In another such family, where no deletion was found, the trait cosegregated with restriction-fragment length polymorphism (RFLP) markers within the dystrophin gene. Van Ommen reported a thorough analysis of 150 deletions that start in the hot-spot region between exons 44 and 45. This corresponds to a very large intron (about 200 kb). Hybridization with whole cosmids covering 100 kb from this intron showed that the breakpoints are widely spread, although clustering appears in some fragments. Thus the propensity of this intron to undergo deletion does not result from the presence of a single unstable sequence. Partial duplications are not infrequent causes of muscular dystrophy and R. Worton (Sick Children's Hospital, Toronto) observed that they often arise on the grand paternal X chromosome as a result of unequal sister-chromatid exchange¹⁴.

Prenatal diagnosis and detection of

carrier females in families at risk is an important issue. At present, this is performed by Southern blot analysis, which is particularly tedious and delicate as for detection of all deletions, eight cDNA probes have to be used (the gene is too complex to be analysed at once with the complete cDNA probe). For carrier detection, because of the presence of one normal gene, a dosage analysis is necessary in the region of the deletion, which is delicate to perform (alternatively, pulsed-field gel electrophoresis can be used to detect an abnormal breakpoint fragment¹⁵). Finally, in the one-third of cases which have no visible deletion, analysis has to be performed with intragenic RFLP markers (and intragenic recombination can cause diagnostic error). J. Chamberlain (Baylor College of Medicine, Houston) reported striking progress on the use of PCR-based strategies to detect deletions in the male patients¹⁶ (amplification of nine regions of the gene allows the detection of about 80 per cent of the deletions in a single reaction) but also gave convincing evidence that this can be applied to carrier detection using carefully designed amplification schemes, fluorescent primers and a modified version of an automatic sequencing machine (the last being a rather expensive item for the average diagnostic laboratory).

Several other strategies were described for the detection of polymorphisms (using allele-specific oligonucleotides or allele-specific primers for amplification of a given allele)¹⁷. Analysis by PCR amplification of the correctly spliced RNA present at very low levels in most or all cell types might also be of diagnostic value¹⁸. Of importance for genetic counselling is the estimate, both by Van Ommen and by U. Francke (Yale University, New Haven) that, because of germinal or somatic mosaicism, the recurrence risk of a new mutation is rather high (about 7 per cent).

E. Hoffman (Children's Hospital, Boston) reported an extensive analysis of dystrophin in muscle biopsies from 370 patients (with Duchenne or Becker forms, or with myopathies with uncertain diagnosis). He concluded that it is the concentration rather than the size of the protein (in deletions or duplication cases) that determines the severity of the evolution: Duchenne patients have no dystrophin, patients with a severe Becker form have usually 3–10 per cent of the normal level of dystrophin, whereas those with more than 20 per cent can be predicted to undergo a mild or moderate evolution.

The astonishing independence between severity and size of dystrophin is illustrated by two extreme examples: a patient with a moderate Becker type described by Hoffman has a duplication of 30 exons and a protein of mass 600,000; Davies reported on a patient with a very mild Becker

form and with a very large deletion that removes half of the spectrin-like region. The possibility to predict the evolution of the disease by protein (or DNA) analysis is important for the large proportion of affected children born in previously unaffected families, and also for constituting homogeneous patient groups for future therapeutic trials. If dystrophin is normal in quantity and quality, then the diagnosis of Duchenne or Becker muscular dystrophy can almost certainly be excluded¹⁹. Conversely, several recent reports show that some patients diagnosed as having spinal muscular atrophy or limb-girdle muscular dystrophy (both autosomal recessive diseases) in fact have deletions in the dystrophin gene^{20,21}.

Animals, models and therapy

A fascinating aspect of the dystrophin problem is the striking difference in phenotype between mice and humans that lack dystrophin. It is well known that the *mdx* mouse has no obvious clinical symptoms and was initially identified by G. Bulfield²² on the basis of increased levels of pyruvate kinase in serum. The pathological findings in the *mdx* mouse were reviewed by G. Karpatis (Montreal Neurological Institute). The *mdx* mouse shows extensive necrosis of muscle fibres from 15 to 80 days after birth, accompanied by active regeneration. Regenerated fibres can be recognized as they have central nuclei whereas normal muscle fibres have peripheral nuclei. After day 80, all fibres seem to be of the regenerated type and necrosis is much less conspicuous afterwards, as if the newly made fibres have become less sensitive to the lack of dystrophin.

One hypothesis would be that in mice a dystrophin-like protein (such as the one encoded by the new gene identified by Love *et al.*¹) could replace the missing protein. This could be reminiscent of the mild phenotype in β -thalassaemia patients who express significant levels of fetal γ -globin. In fact, the *mdx* mouse has more muscle mass and appears stronger than the normal mouse! In contrast, in man the regenerative potential is rapidly exhausted by the continued necrosis. H. Blau (Stanford University), who has devised a method to prepare pure muscle satellite-cell populations, showed that even just a few months after birth, the replication potential of these cells (mononucleated myoblasts which can participate in regeneration and formation of new fibres) is less in Duchenne patients than in an older normal control, and at the age of 4 the replication potential is almost exhausted (less than 10 doublings). The dystrophic dog has a disease very similar to that of man, but heterogeneity of clinical evolution in the same sibship was reported²³.

Because of the high rate of new muta-

tions in the dystrophin gene, genetic counselling and prenatal diagnosis will have only a limited effect on the incidence of the disease, and the design of efficient therapies is thus a major concern. The problem seems a formidable one because dystrophin is important in the structure of both skeletal and cardiac muscles. The demonstration by T.A. Partridge *et al.*²⁴ that injection of myoblasts from normal mouse into growing or regenerating muscle of *mdx* mouse can lead to substantial expression and correct localization of dystrophin in muscle fibres, in the injected muscle, generated considerable interest and hope in patients' associations. However, both in his paper and at the meeting, Partridge (Charing Cross and Westminster Medical School, London) stressed the numerous problems that should be dealt with for therapy in man: immune rejection (the successful implantation experiments were performed in immunodeficient *mdx*/nude mice); the production of a very large number of myogenic cells; the necessity of injection at multiple, probably closely spaced sites because dystrophin, like other muscle proteins²⁵ remains localized, within the fibre, close to the nuclei from which it is derived. Other approaches might be tested in subgroups of patients, for instance trying to boost expression of dystrophin in severe Becker patients. And Kunkel concluded in a rather optimistic mood that various therapeutic avenues might now be explored. □

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Boring and crazing

To propagate through a solid, a crack must find from somewhere the energy needed to form its growing surfaces. If its surface-energy were ever zero, a crack could spread and ramify spontaneously. This actually happens when water is forced into a viscous material with which it is thermodynamically miscible. A convoluted aqueous intrusion of fractal complexity is formed.

This principle, says Daedalus, has powerful implications for geotechnology. What would happen to a borehole if a liquid pumped into it had zero interfacial energy with the local rock? The pressurized liquid would effortlessly penetrate and extend all the microcracks in the rock, crazing them into sub-cracks and sub-sub-cracks as it went. The result would be a sort of fractal tree root structure, radiating out in all directions from the borehole.

DREADCO's chemists initially recommended hydrofluoric acid for this job. It actually dissolves most rocks with evolution of heat, implying not merely a zero interfacial energy, but one strongly negative. But Daedalus distrusts this most corrosive and treacherous of acids, and prefers liquids that only just dissolve rock, or merely resemble it so closely that the interfacial energy vanishes. For silicate-based rocks and clays, compositions of hydrofluosilicic acid and sodium silicate solution ('water glass') seem promising.

When perfected, the new borehole technology will have wide and wonderful applications. Just bore your hole, line it, and pump a suitable DREADCO fracture fluid into it. From the bottom of the hole a fractally convoluted web of cracks and crevices will spread outwards until every element of the local rock is within an arbitrarily short distance of a crack. If the rock is water-bearing, the result is an ideally efficient well. If it is dry, the hole would make a perfect soak-away drain or wastewater-disposal pit. And if it contains valuable minerals, such holes provide a perfect way of getting at them. Many metals occur at such low concentration in an ore body that open-cast mining is very wasteful. A few holes bored into the deposit, and pressurized till their fracture trees intersect, will do the job much more neatly. An ore solvent pumped down one hole and extracted from its neighbour will travel a fractally complex path, passing arbitrarily close to every particle of ore. With no environmental damage, this elegant mine will swiftly extract all the ore in even a very dilute deposit.

Fracture fluid will even help to bore the initial holes; as a drill lubricant it will reduce the power needed and heat generated almost to zero. A version matched to the surface-energy of teeth could thus bring about a humane revolution in dentistry.

David Jones

Neu receptor dimerization

SIR—The probable mechanism by which the binding of the (as yet unidentified) ligand to the rat growth factor receptor encoded by the *neu* gene (which is closely related to the gene for epidermal growth factor receptor) is that binding promotes receptor dimerization, which activates the cytoplasmic tyrosine kinase (TK) domain of the Neu receptor¹. Starting from the observation that mutations that convert the valine residue at position 664 (Val 664) of the Neu receptor into glutamate, glutamine or aspartate result in the constitutive activation of TK, and hence the conversion of *neu* into an oncogene that transforms cells, our molecular modelling now explains the effect of the mutations in terms of the dimerization of the transmembrane α -helix of the Neu receptor. Very recently, Weiner *et al.* have shown that the presence of Glu 664 in Neu leads to receptor aggregation².

We have previously proposed³ a model for aggregation based on the formation of a dimer of Neu receptor transmembrane α -helices. Our more recent stereochemical modelling considered the following sequences:

Residue	661	662	663	664	665
<i>neu</i> oncogene	-Ala-Thr-Val-Glu-Gly-				
<i>neu</i> gene	-Ala-Thr-Val-Val-Gly-				
Position(P)	0	1	2	3	4

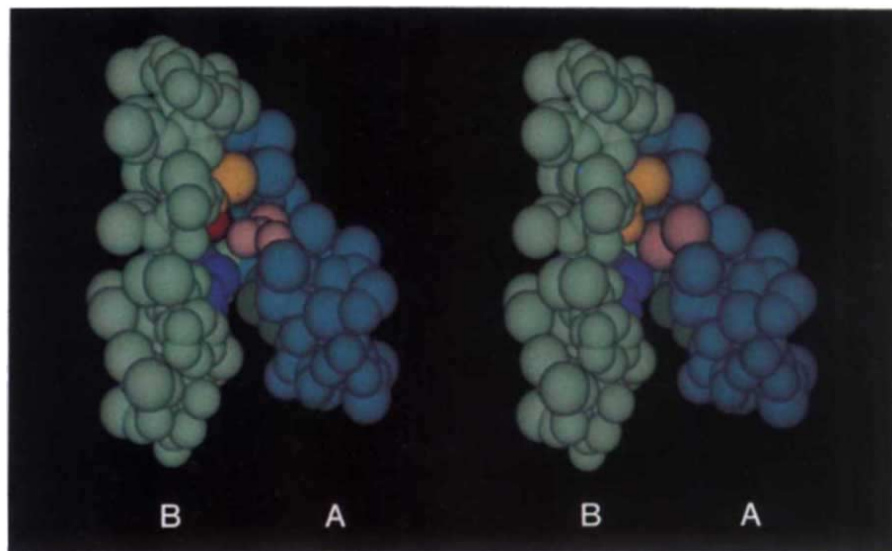
In the hydrophobic membrane, Glu and Asp side chains in oncogenic Neu will be protonated and may form a hydrogen bond with the other helix, stabilizing dimerization. One possibility³ is that the Glu side chains are extended and form carboxyl-carboxylate hydrogen bonds. An alternative interaction involves a symmetric helix packing in which the carboxyl group of Glu 664 in one helix (helix A, left-hand figure) forms a hydrogen bond with the carbonyl oxygen of Ala 661 in the other (helix B, left-hand figure). The carbonyl oxygen of Ala 661 still forms the main-chain hydrogen bond in α -helix B. A second, symmetric hydrogen bond is formed between Glu 664 in B and the oxygen of Ala 661 in A. The helical axes lie at about -50° , a favoured orientation⁴, permitting extracellular/extracellular and intracellular/intracellular domain associ-

ations. In normal Neu (righthand figure) the same transmembrane helical arrangement would pack Val 664 (P3) in A against Gly 665 (P4) in B. The helix packing is mediated by small side chains at P0 and P4.

Enhanced dimerization of the *neu* oncogene product, which may mimic the aggregation of growth factor receptors induced by their ligands, probably accounts for its constitutive TK activation. Activation can be explained stereochemically. Two inter-helical hydrogen bonds can be formed in the Glu mutant of Neu, promoting dimerization and higher TK activity. In normal Neu, the same

Asn might lead to increase in TK activity in some of these receptors. In all 17 reported DNA sequences, a single base change at P3 could produce one of these mutations. Indeed, in the epidermal growth factor receptor the mutation of Val to Glu at P3 leads to some increase in transforming activity, although this requires the presence of the ligand⁵. However, our model for Neu dimerization involves a highly specific inter-helical interaction and may not extend to other receptors.

It will be interesting to test whether peptides corresponding to the Neu transmembrane region, particularly those with a transforming mutation, can specifically inhibit TK activity by forming a non-



Space-filling models for the packing of part of the transmembrane α -helices in Neu. In both cases, the left helix (B) is mainly in light green, the right (A) in light blue. In helix B, Ala 661 is in yellow, Gly 665 in dark blue. In helix A, Glu 664 is in pink forming the proposed hydrogen bond with the carbonyl oxygen (red) of Ala 661 of B. Right, normal Neu with Val 664 in helix A in pink packing against Gly 665 in B.

packing can occur but this is stabilized only by van der Waals forces leading to fewer dimers and lower constitutive TK activity. The Gln and Asp mutations may form weaker inter-helical hydrogen bonds consistent with the lower levels of activity observed. A similar model would explain why Glu and Asp mutations at the equivalent position in *c-erbB-2* (the human equivalent of *neu*) are also activating⁶.

Helix association may also be a component of TK activation in other growth factor receptors. In 18 of the 20 transmembrane regions of such receptors⁶⁻⁸ we have examined, we find a sequence motif analogous to that required for the packing in Neu. P0 requires a small side chain (Gly, Ala, Ser, Thr or Pro); P3 an aliphatic side chain (Ala, Val, Leu or Ile) and P4 only the smallest side chains (Gly or Ala). From the observed residue frequencies in the 20 transmembrane regions, the probability that the pattern occurs by chance in 18 out of the 20 sequences is only 0.0005.

Mutations at P3 to Glu, Asp, Gln or

productive complex. If so, and if dimerization is a more general phenomenon, such peptide inhibitors may represent a novel therapeutic strategy for cancer cells whose transformed state is dependent on unregulated growth factor receptor activity.

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Erratum

In the Scientific Correspondence by William Fowler on cold fusion in the 1 June issue of *Nature* (p.345), the 4th line of the second paragraph should read: "Nevertheless, I prefer to express my results in terms of transition widths. . .". The 5th line from the end of the same paragraph should read: ". . . $S_2 \rightarrow D_0$, of the nuclear states. . .".

On the same page, equation (4) should read:

$$\Gamma_n = \frac{1}{375\pi} \alpha^2 Z^2 \frac{E_n^5}{(\hbar c)^4} a_n^4 f_n$$

Learning by handshake?

SIR—We recently reported that long-term potentiation (LTP) in some hippocampal synapses is associated with a slow increase in the sensitivity of CA1 neurons to analogues of the neurotransmitter L-glutamate¹. This result implies that post-synaptic mechanisms can underlie the long-lasting enhancement of synaptic efficiency in this experimental model of learning. Because LTP is generated within seconds, yet the postsynaptic change takes about an hour to reach its maximum, we suggested that presynaptic mechanisms account for the early phase of LTP. On the basis of these data, and two other recent publications^{2,3}, Stevens⁴ suggested in his News and Views article a four-step mechanism for the generation of LTP (see his figure in ref. 4). Stevens proposes that a complex sequence of intercellular messengers provides a 'handshaking' mechanism whereby checks are instituted to ensure that the necessary conditions are met for having an appropriate increase in synaptic strength.

Although such a complex scheme is possible, we wish to point out that it is not necessary to account for the data¹⁻³. An alternative explanation is that the post-synaptic calcium influx triggers the post-synaptic change and that the delay is inherent in the sequence of biochemical processes involved.

We believe that the increase in sensitivity involves protein kinases, because we find that the increase is blocked by the potent protein kinase C inhibitor K-252b (unpublished observations). Because manipulations of the postsynaptic cell seem to block LTP from its onset, there still seems to be the need for a retrograde messenger.

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Mystery of meteoritic fusion

SIR—Although the concentrations of radioisotopes generated by cosmic rays in recently fallen stony and iron meteorites are in agreement with expectation, it has been known for several years that tritium is exceptional in that its concentration in iron meteorites is deficient¹.

The usual explanation is that tritium diffuses rapidly out of iron meteorites^{2,3}, but this is contradicted by measurements of helium-3, a decay product of tritium. Both the concentration of helium-3 and the helium-3/helium-4 ratio indicate that tritium has been present throughout the radiation history of the meteorites in the amounts expected from known cross-sections, and that it must decompose within the meteorites.

Some years ago, I put forward^{4,5} the radical alternative explanation that tritium in a metal matrix no longer decays with a 12-year but with a much shorter half-life. This suggestion has been vigorously criticized (see, for example, ref. 6), but the low content of tritium in iron meteorites remains a puzzle, at least to me.

The recent reports^{7,8} that deuterium fusion can occur rapidly in a metal matrix are also puzzling. Deuterium–deuterium fusion may either lead to tritium plus hydrogen or helium-3 plus a neutron, but relatively few neutrons have been observed and also little tritium. At first sight, this

would exclude both deuterium reactions.

I would like to suggest that the reaction leading to tritium is predominant, but that the isotope is detected in only small amounts because it decays rapidly, in the metal matrix, to helium-3. The experiment suggested as a test of the hypothesis that tritium decays rapidly in iron meteorites would also apply in the deuterium fusion experiments — to look for a rapid growth of helium-3.

I acknowledge that the suggestion that the half-life of tritium can be lowered in a metal matrix is contradicted by the well-established theory of β -decay, but the low concentration of tritium in iron meteorites and the small quantities of neutrons and tritium found in the deuterium fusion experiments are both so puzzling that the possibility that some unanticipated effect is affecting the decay constant should not be excluded.

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Radiation risk

SIR—Dunster¹ argues in favour of radiation protection standards based on detriment rather than risk. Ideally the setting of standards is a biphasic process; first, a level of risk or detriment deemed to be acceptable should be set, then the doses and exposures that equate to this risk should be derived. The first phase is a political judgement, the second a scientific interpretation of available data, in which scientific rigour has to be compromised by the need for utility.

The existing framework recommended by the International Commission of Radiation Protection (ICRP)², and accepted in the United Kingdom, deems acceptable for radiation workers the risks associated with 'safe' industries. The process of defining the relationship between risk or detriment and dose is fraught with uncertainty. Dunster argues in favour of detriment on two main counts: first, projected years of life lost to attributable causes is more relevant than deaths; and second, latency defers the risk to later in life even to the point that it may not be expressed.

It is true that 'years of life lost' is less sensitive to risk projection models than is risk itself, but the factor 2 involved is less than the uncertainty involved in inferring risks using data from the best available

epidemiology³. Indeed the UNSCEAR report³ is at considerable pains to point out the uncertainty in these estimates. Add to this the uncertainty involved in extrapolating to low doses and low dose-rates (UNSCEAR³ proposes a value between 2 and 10) and a factor 2 becomes inconsequential. The ameliorating effect of taking latency into account is small.

The demands of utility, at least in the present system recommended by the ICRP², also distort the scientific basis in several ways. For example, the risk of breast cancer induction by radiation is negligible except for women exposed under the age of 40 — about a quarter of the population — and yet this risk is deemed to be averaged over the whole population.

Thus risk, in relation to radiation-induced cancer at low doses and dose-rates, is intrinsically very uncertain; the need to use it in a workable framework of protection standards inevitably devalues it further. In expressing the harmful effects of radiation, careful consideration should be given to the degree of refinement that it is legitimate to use.

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Chemistry in industry

David Fishlock

Science and Corporate Strategy: Du Pont R & D, 1902–1980. By David A. Hounshell and John Kenley Smith, Jr. Cambridge University Press: 1989. Pp. 756. £27.50, \$39.50.

IN April 1930 Du Pont made two remarkable discoveries in the new science of polymer chemistry. They led to the products nylon and neoprene — which, as 1990 approaches, are still earning profits for the company.

Nylon in particular provides a model for successful research and development, and not only because its revenues are an order of magnitude greater than for neoprene. In this book, two business historians, who were invited to examine the role of research and development in the evolution of the biggest chemicals company in the United States, show how the management concentrated scientific resources upon clearly identified targets, and hit them every time with the kind of success we associate nowadays with Japan.

Few scholars, the authors testify, could hope for a subject offering richer rewards or better questions to address. They do full justice to all the opportunities — and the freedoms — Du Pont offered. Their tale is a long one spanning nearly 80 years, but they have a light touch and their lessons are leavened with countless anecdotes that will be enjoyed by every

research manager. In every sense they have written the story of twentieth-century science in Du Pont.

"Nylon was an unprecedented and as yet unrepeatable success for Du Pont" the authors conclude. Julian Hill, attempting to produce molecular chains longer than anyone had managed before, happened upon a polymer filament that when cool would stretch into an amazingly strong fibre. It had a molecular weight exceeding 12,000 — far beyond that of any condensation polymer previously prepared. Hill was part of a fundamental research programme led by Wallace Hume Carothers, who was recruited in 1928 from Harvard, and who in his nine years with Du Pont became the foremost organic chemist in the United States.

But crucial also to the development of nylon (and neoprene) was the promotion of Elmer Bolton to chemical director in June 1930. Bolton, also a brilliant organic chemist, had been a key figure in the company's long and difficult struggle to become a profitable dyestuffs producer. He had become "a commercially oriented, pragmatic research director" with a

strong belief that research could be managed like any other part of Du Pont's business. Bolton was suspicious of the kind of long-range research being pursued in the fundamental research programme, and had opposed its creation. Now he was put in charge.

These abrasive ingredients — academic brilliance and pragmatic concern with real products — brought nylon galloping out of the laboratory and into the market in only five years. The scientists had ideas for using nylon to replace a host of materials, such as cellophane, photographic film, leather and wool, but the company picked a single target: the silk stocking, seen by American women in the 1930s as "the symbol of liberty, democracy and undisputed self-respect". The company also focused on a single process for each stage of the complex manufacturing cycle. This, too, was a risky strategy, but it succeeded, in the words of one executive, through "brute force and awkwardness", in undercutting the price of silk.

By the mid-1930s, Hill's original fibre had burgeoned into 81 prospective polyamide compounds from which Bolton picked the 6-6 polymer, starting from the relatively cheap feedstock benzene. "In retrospect", Hounshell and Smith say, "the development of nylon appears to be the solution of thousands of small problems; but this kind of engineering could begin only after the big decisions were made about how nylon was to be manufactured". The big decisions were taken

"How soon, Mr Chemist? And the chemist answers 'Just as soon as I can make it come true. I build for the tomorrow that will be yours, and your children's and your grandchildren's. And when each of these tomorrows become a 'today' there will still be tomorrow to work for'."

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

The public's first view of nylons at the New York World's Fair in 1939. Above is an extract from Du Pont's ebullient advertising of the period.

early and were not questioned when the project hit problems: "Generally, the development of nylon proceeded in a systematic and orderly way". In the book, the "nylon model" is described in painstaking detail, covering three phases of development: commercial feasibility of the new fibre; the practicability of producing high-quality yarn to compete with silk for fully fashioned hosiery; and the reproducibility of this yarn on a very large scale.

Du Pont launched its yarn in 1938 with the claim that "from such common raw materials as coal, water and air, nylon can be fashioned into filaments as strong as steel . . .". This inspired the *New York Times* headline, "New hosiery as strong as steel", and ideas that such stockings would never wear out. Soon it was also inspiring hucksters to sell silk stockings as nylons, surely the hallmark of success for the project.

But Du Pont failed to apply the "nylon model" to Corfam, a man-made alternative to shoe leather, a goal from the second decade of corporate research and development which was realized in 1949 with the discovery of a new approach to porous non-woven fabrics. Over the next five years three different departments spent over \$2 million trying to develop nylon-based compositions before the company ruled that one alone should carry the development forward.

In 1958, the department came up with a new material based on Dacron, another Du Pont invention. It was a composite structure consisting of a non-woven Dacron substrate with a porous polyurethane binder and a porous outer coating. Four years later the company distributed 15,000 pairs of Corfam shoes for 'clinical trials'. Complaints were sufficiently few to encourage the idea that, as with nylon, it had a product superior to the natural one. It was unaffected by moisture, weighed one-third less, kept its lustre and did not need to be broken in.

Corfam came to the market in 1964, 15 years after the original invention, but still saddled with an "overly complicated" manufacturing process that obliged the company to compete in the market for top-quality shoes. Although 45 million pairs of Corfam shoes were sold in the United States over the next five years, the material failed to show a profit. During this period imports of real leather shoes were rising rapidly, forcing Corfam to compete downmarket. When it was finally abandoned, the material had accumulated venture losses of about \$70 million. Corfam, once heralded as a new nylon, became known as Du Pont's Edsel, with little evidence that the hard-won lessons of the "nylon model" were ever seriously applied. □

David Fishlock is Science Editor of the Financial Times.

Out of focus

Roger Taylor

The History of Photography Vol. II. The Rise of Photography 1850–1880: The Age of Collodion. By Helmut Gernsheim. Thames & Hudson: 1989. Pp.285. £40, \$65.

IN 1939 some notable books on photographic history were published, their appearance being primarily due to the centenary of photography's announcement. Fifty years later, as we celebrate the 150th anniversary, another surge of publishing is underway to mark the event.

During the intervening years there has been a great change in the status of photographic history. Gradually it has emerged from being a marginal subject pursued by a few, and has entered with force and vigour into cultural life generally. That transformation was marked by a number of events, none more important than the publication of *The History of Photography* by Helmut and Alison Gernsheim, first in 1955 and then in enlarged and revised form in 1969. This second edition became the standard reference work for everyone interested in the subject.

As the research of other photographic historians began to accumulate, however, there was a realization that the Gernsheim's text was often based upon secondary sources and the reminiscences of others. Implicitly this was always recognized to be the case, and was accepted—it would have been impossible to assemble such a comprehensive volume and substantiate every fact and detail through primary research. Now we have the completion of the third edition of their work, which has been published in two volumes. The first, *The Origins of Photography*, appeared in 1983. The second, reviewed here, deals with the period 1850–1880, "The Age of Collodion".

During the 20 years between editions, the pace of research on photographic history has accelerated, and there have been countless books and articles shedding new light and correcting former mistakes. Historians have concentrated upon specific individuals, processes or aesthetic movements to enrich our understanding of the development of photography through the years. In both volumes of this new edition, one is aware of Gernsheim's selective interpretation of the fresh historical evidence. In some cases the very absence of such information, even though it is readily available, is something of a shock.

The example of Gustav Le Gray will illustrate the point. Le Gray was an important mid-nineteenth-century photographer whose impressive seascapes have consistently been admired. His technical

achievements have been debated widely by photographic historians, with the discussion centring on his ability to harness technique in the service of art. In her monograph *The Photography of Gustav Le Gray*, published by University of Chicago Press in 1987, Eugenia Parry Janis describes all his working methods and refers in detail to the various devices he used to achieve results. Gernsheim does not even mention this publication; instead he refers to a much earlier article from 1980, which he selectively quotes from in order to support his original views on Le Gray's techniques, as related in the second edition.

This lack of precision leads to confusion and a compounding of the problem about the very nature of photographic history. It is a truism that an error repeated frequently enough transforms itself into a fact. The latest victim to fall foul of this is Asa Briggs in his book *Victorian Things* (Batsford, 1988), which inevitably has photography as one of its themes. Much of the information and interpretation has been taken from secondary sources, including *The History of Photography*, and repeated within a different context. The text is littered with errors, but stated so convincingly that they are credible. No doubt, in time, Briggs will be quoted as a source and the mythology of history will become woven more intricately than ever.

My criticisms of *The History of Photography* extend, I fear, beyond the text to the illustrations. Photographs are used in many ways. Most frequently they present the visual evidence of the past, a tangible record of existence, which places them in the role where content is all important. In a book dealing with the history of photography it is vital to treat the photographs both as object and subject, and respect their original context and integrity. So it is a great pity that in Gernsheim's book the photographs have been cropped and altered to fit the designer's ideals; for example photographs that were originally vertical now appear as square or horizontal, a fact made all the more puzzling by the inclusion of note of the original dimensions.

Sadly, Gernsheim and his publisher have missed the opportunity to revise a seminal yet preliminary account into a modern work of reference. Other photographic histories, monographs and articles must now fulfil that role. □

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● Academic Press has published the third volume (out of a projected four) of *Geomagnetism*, edited by J. A. Jacobs. The book contains seven chapters, which are divided in subject matter between the Earth's main magnetic field and aeronomy. Price is \$130, £65. Volumes 1 and 2 were reviewed in *Nature* 332, 596 (1987).



Making faces — facial markings of the cheetah, which are used to emphasize expression of confidence, aggression, submission and the deflection of threatening advances. The illustrations are taken from the new paperback edition of Jonathan Kingdon's *East African Mammals Vol. IIIA: Carnivores*, published by University of Chicago Press. Volumes IIIB-D are also now in paperback.

Field work

Sean Washburn

Magnetoresistance in Metals. By A. B. Pippard. Cambridge University Press: 1989. Pp.253. £39, \$59.50.

PROFESSOR Pippard has expanded on his earlier monograph (*Dynamics of Conduction Electrons*) in a new book that surveys the broad topic of magnetoresistance in metals. A chapter on fundamental aspects of transport in a magnetic field, pitched at the level of Ziman's *Principles of the Theory of Solids*, introduces matters, preceding a review of theoretical and experimental work. Classical magnetoresistance is discussed along with illustrative experiments, and then 'semi-classical' effects are surveyed in detail. Untangling of this web of data takes up the bulk of the text, and it is no mean feat; for example even potassium, "the simplest of metals" which has a nearly spherical Fermi surface, exhibits a bewildering variety of magnetoresistance. Finally, Pippard turns to inhomogeneous samples and briefly describes recent results, including electron focusing and resistance fluctuations arising from Aharonov-Bohm effects and coherence of the electrons.

The book proceeds from the simple to the complex; one starts from a familiar base and then is led to attempt interpretation of a great deal of complicated data.

The text is accompanied by diagrams that define notation and illustrate the physics, and by representative data from experiments. Here as in other texts, the depictions of Brillouin zones would have benefited from colour and modern computer graphics. The physics is always deep enough to describe the data and is clearly presented. It is, however, never more complicated than a single particle picture (implicitly from the Fermi liquid) — interaction among the electrons is mentioned only casually in connection with heavy-fermion metals.

Does this book meet a need? Yes. In particular the chapter on experiments should be read by anyone attempting to make such measurements, because here Pippard lays down clear warnings about spurious responses and describes methods that avoid them.

I have a few complaints. There is disproportionate citation of work performed in Britain, even after allowing that much of the original research was carried out at the Cavendish Laboratory. There are also quite a few annoying typographical errors (mostly obvious, such as the mislabelling of figures), and occasionally the language and notation are unusual. Nonetheless the book will serve well as background reference for anyone working in this field, and as a concise introduction for anyone entering it. □

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Bringing it all together

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Biomembranes: Molecular Structure and Function. By Robert B. Gennis. Springer-Verlag: 1989. Pp.533. \$59, £32.

ROBERT Gennis's *Biomembranes* is the fourth volume to appear in Springer's *Advanced Texts in Chemistry* series. The series has an established record of excellence, and this latest addition lives up to expectations. Gennis set out to produce a book that he would like to have had as a student, as well as one that he would find useful as a research scientist. He has achieved both of these goals, bringing together the diverse areas of membrane biology in a manner that is both informative and stimulating.

Here is perhaps the most complete treatment of membrane biology currently available. It covers almost all aspects of the subject, not only structure but also transport, receptors and biogenesis — there is something for everyone. Indeed, the author apologizes for the possible inclusion of too much information, and suggests that readers may wish to skip over sections in which they are not interested. The apology is unnecessary. Although I do take Gennis's point, his intention has been to arrange the material to the best effect for teaching purposes. Thus there is a gradual introduction to the structure and composition of membranes, Chapter 1 providing a general survey of the various lipids and proteins, Chapters 2 and 3 dealing with them in greater detail, and Chapters 4 and 5 describing their interaction.

Features of the book I especially liked are the summaries at the end of each chapter — they are true summaries, uncluttered with detail — and the numerous 'boxes' which are used to highlight certain aspects or to cover a particular point in depth without distracting from the flow of the main text. The reference list is a highlight, being comprehensive yet unobtrusive.

My only criticism concerns the figures. Although most of those that do appear are relevant and clear, the book would have benefited from having more of them, and from the inclusion of photographs. Students now expect their textbooks to contain an abundance of high-quality illustrations, and the comparative lack of visual material will, I suspect, lessen the acceptance of *Biomembranes* for teaching. Given its price, though, this is a book that is likely to be bought largely by libraries and laboratories. It is also one that will be in great demand. □

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Coming out of Africa

David J. Chivers

A Primate Radiation: Evolutionary Biology of the African Guenons. Edited by Annie Gautier-Hion, François Bourlière, Jean-Pierre Gautier and Jonathan Kingdon. Cambridge University Press: 1988. Pp. 567. £60, \$120.

AFRICA's primates have been the subject of intensive research for the past 20 years, yet books bringing together the results of the various studies have been conspicuous by their absence. Unlike their relatives in the rainforests of south-eastern Asia and the Neotropics, African primates occur in a great variety of habitats. They exhibit a bewildering complexity of taxonomy, distribution, phylogeny, biology and socioecology — and none more so than the members of the genus *Cercopithecus*, the guenons.

The situation is much clarified in *A Primate Radiation*. The book contains wide-ranging analyses of the 28 species (seven superspecies) and about 75 subspecies arranged in four subgenera — *Erythrocebus* (the patas monkey), *Miopithecus* (the talapoin monkey), *Allenopithecus* (Allen's swamp monkey, demonstrated to be closer to the ancestral guenons than the 'true' guenons) and *Cercopithecus* (the guenons, the other 25 species).

The editors have done an excellent job, dividing the material into three well-integrated sections: guenons and the African environment, past and present (six chapters); genetic and phenetic characteristics, and their use in phylogenetic reconstruction (eight chapters); and ecology and social behaviour (ten chapters). This last section includes both fresh data and stimulating new analyses. A picture emerges of behavioural flexibility in diverse habitats, of one-male groups (Cords), eating mainly fruit (Gautier-Hion), breeding seasonally (Butynski) with some promiscuity (Cords, Struhsaker), and maintained by more subtle means than the frequent and overt signals of baboons and macaques (Rowell). The occurrence of mixed troops (Gautier-Hion) is of key importance in this context, as they reduce predation and increase efficiency of foraging on diverse plants with complex production patterns. Mixed troops also help to promote hybridization (Struhsaker *et al.*), and this may be of long-term significance in the speciation and subspeciation of guenons.

In the preceding sections, the contributors emphasize the dichotomy between the savannah or more terrestrial forest species and the more derived arboreal

forest species, as well as their biological plasticity. A strength of the book and its science is the concordance of results from diverse areas of research. Thus studies of fossils (Meave Leakey), of forest evolution (Hamilton), of habitat and locomotion (Pickford and Senut) and of present-day distribution (Lernould, Oates, Colyn) all support the idea of a recent radiation of forest species (in the past five million years) from Miocene semi-terrestrial frugivores. Guenon systematics and phylogeny are further clarified genetically by the protein electrophoretic studies of Ruvolo and Turner *et al.*, and by the detailed chromosome studies of Dutrillaux *et al.*; and phenetically by the morphological studies of Turner *et al.*, Martin and MacLarnon, and Kingdon, as well as through detailed analyses of calls (Gautier).

François Bourlière has inspired several generations of tropical ecologists, and evidence of his broad and incisive mind is clear throughout the book. The Gautiers have pioneered and persisted in the study of elusive west African forest primates, while their French colleagues have developed new laboratory approaches. The art work and biological vision of Jonathan Kingdon is an added bonus. The four editors, 26 other contributors and the publisher have completed an impressive task, producing a well-coordinated and attractive volume. *A Primate Radiation* will long stand as the key source on African primates and as a model for further multidisciplinary research on any continent. □

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Andromeda and beyond

Richard McMahon

Extragalactic Astronomy. By B. A. Vorontsov-Vel'yaminov. Translated by Richard B. Rodman. Harwood: 1988. Pp. 724. \$285, £187. Book club price \$85, £56.

"Of all the objects located beyond our Galaxy, only one, the great spiral nebula in Andromeda, can be glimpsed by the unaided eye in the northern celestial hemisphere." Thus begins *Extragalactic Astronomy* by Professor Vorontsov-Vel'yaminov of the Sternberg Astronomical Institute, Moscow University, one of the Soviet Union's foremost observational astronomers. Although the existence of the Andromeda Nebula had been known for more than 1,000 years, it was not until the 1920s that its true nature became clear with the realization that, just as the Sun is merely one star out of the many millions in our own Galaxy, the Milky Way itself is just one of many millions of galaxies. With this realization of the immensity of the Universe, a new branch of astronomy was born: extragalactic astronomy.

Vorontsov-Vel'yaminov is probably best known for his *Atlas of Peculiar Galaxies*, published in 1959. In that work he drew attention to the observation that the shapes of many galaxies do not fit very well with the well-known categories of spiral and elliptical proposed by Edwin Hubble, showing that the Hubble classification is an unrealistic oversimplification. Most galaxies are perturbed or deformed, and many appear to be interacting with their neighbours. The effect of interactions in galaxy formation and evolution

remains an important area of research.

Vorontsov-Vel'yaminov's publications in professional journals span half a century, and in this book he has provided us with a fine systematic survey of extragalactic astronomy. The English version of the book is a translation of the second Russian edition. Although that was completed in 1978, the translation is supplemented with an appendix by Vorontsov-Vel'yaminov himself, covering the period up to 1982, while the period since then is dealt with by Debra Meloy Elmegreen. So although there has been a considerable delay between the original Russian publication and the appearance of the English translation, the contents of the book are completely up to date. A particularly valuable ingredient is the final bibliography, which contains nearly two thousand references which all readers will find an invaluable source for further exploration of the immense amount of published material on observational extragalactic astronomy. Moreover, as one would hope, the work of Soviet astronomers is cited more than it would be in a comparable account of the subject published in the West.

The book emphasizes observation rather than theory, which means that the contents will not date so much as would those of a theoretical treatise. This does not mean that theory is neglected, but that the treatment of the observations is based more on qualitative physical arguments than on a detailed comparison with the theory of the day. As a result, however, the text is rather long-winded in places.

This book is more than just a translation. Using the basic Russian text, the production team has produced an up-to-date review of observational extragalactic astronomy.

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Protein encoded by *v-erbA* functions as a thyroid-hormone receptor antagonist

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The thyroid-hormone receptor can, in the absence of its ligand, suppress activity of a responsive promoter. Addition of thyroid hormone, however, results in the stimulation of expression. The oncogenic derivative of the thyroid-hormone receptor, *v-erbA*, acts as a constitutive repressor and, when coexpressed with the receptor, blocks activation by thyroid hormone. Thus, *v-erbA* may be the first example of a dominant negative oncogene.

THE search for the genetic basis of cancer has led to the identification of a group of genes whose products contribute directly to cell growth, differentiation and development. These cellular genes, termed 'proto-oncogenes', however, seem not to be tumorigenic themselves, but rather must be activated by mutation and/or abnormal levels of expression¹. An important advance was the recognition that these genes can be classified as either dominant or recessive depending on whether the presence or absence of their product contributes to tumour development and progression².

A variety of experiments have demonstrated that two oncogenes may exert a synergistic effect on cell transformation³. In fact, the transforming capacity of some oncogenes is manifested only in the presence of another transforming entity. One such example is the *v-erbA* gene, found in the avian erythroblastosis virus (AEV), which strongly potentiates the transforming activity of a number of tyrosine kinase and *ras*-related oncogenes⁴⁻⁶. It has been suggested that *v-erbA* expression contributes to erythroleukaemia by interfering with the programmed differentiation of erythroid precursor cells⁴⁻⁷.

The similarity of the *v-erbA* oncogene product to the glucocorticoid receptor ultimately led to the identification of its cellular homologue as the thyroid-hormone receptor^{8,9}. We now recognize the existence of a superfamily of ligand-inducible transacting factors, including those for steroid hormones, retinoic acid and vitamin D3, as well as two subtypes (isoforms) of thyroid-hormone receptors, termed α and β ¹⁰. Mutational analysis and structural comparisons of these hormone receptors identified domains responsible for hormone binding, DNA binding and *trans*-activation of gene expression¹⁰. As with the steroid receptors, transcriptional activation by the thyroid-hormone receptors is dependent on the presence and binding of the respective ligand¹¹. But deletion analysis showed that glucocorticoid, oestrogen and progesterone receptors lacking the hormone-binding domain still recognize the specific response elements and may function as constitutive activators¹²⁻¹⁸. Thus, neither the ligand itself nor its binding domain needs to participate directly in DNA recognition.

The *v-erbA* protein contains an apparently intact DNA-binding domain. As a result of amino-acid changes and a deletion in the C-terminus, however, it has lost its ability to bind thyroid hormone^{8,19}. By analogy to the mutated steroid-hormone receptors, we have proposed that these mutations, in conjunction with the high level of expression, convert the thyroid-hormone receptor into a hormone-independent transcription factor^{8,9}.

We therefore initiated experiments to analyse the functional

properties of the oncogene and its cellular homologue. Using a gel-retardation assay, we show that both *v-erbA* and the thyroid-hormone receptor recognize and bind to a cognate response element in the absence of ligand. The consequence of this binding is a suppression of a thyroid-hormone-responsive reporter gene by the non-liganded receptor. Addition of thyroid hormone results in a 10- to 100-fold stimulation of transcription from the repressed level. Surprisingly, *v-erbA* does not function as an activator, as speculated, but rather as a constitutive repressor of triiodothyronine (T_3) responsive genes. When coexpressed with the thyroid-hormone receptor the *v-erbA* repression is dominant, demonstrating that *v-erbA* can function as a potent receptor antagonist.

Biological activity of rTR α and *v-erbA*

The transcriptional activity of both the rat thyroid-hormone α -receptor (rTR α)²⁰ and the *v-erbA* oncogene product (Fig. 1a) was assessed by their ability to regulate the expression of thyroid-hormone-responsive reporter genes. These constructs contain oligonucleotides corresponding to previously identified thyroid-hormone response elements (TRE)^{21,22} linked to a *tk*-CAT (thymidine kinase-chloramphenicol acetyltransferase) fusion gene²³ (Fig. 1b). Expression plasmids containing rTR α com-

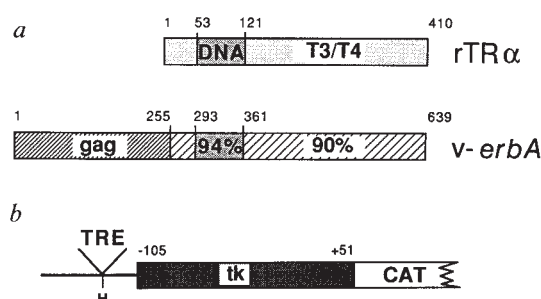


FIG. 1 Expression and reporter constructs. *a*, Schematic organization of the rTR α and *v-erbA* protein sequences. cDNAs encoding these proteins were cloned into an RSV expression vector. DNA and T_3/T_4 refer to the DNA- and thyroid-hormone binding domain, respectively. The 12 amino-terminal amino acids of chicken *c-erbA*/TR α are replaced by part of the retroviral *gag* gene product, resulting in the synthesis of a P75^{gag-v-erbA} hybrid protein. In addition, *v-erbA* differs from chicken *c-erbA*/TR α in two amino acids in the DNA-binding domain and 9 amino acids plus a 9 amino-acid deletion in the hormone-binding domain. The comparison to rat TR α shown here reveals additional 17 amino acid differences that are species specific and also found in comparison between chicken and rat TR α deduced amino-acid sequences. Numbers on top indicate amino-acid positions. *b*, The reporter gene constructs used contain oligonucleotides encoding the respective T_3 response elements (TRE) inserted into the *Hind*III site (H) upstream of the *tk* promoter-CAT construct.

METHODS. Construction of the expression vector RS-rTR α has been described²¹. The expression plasmid RS-*v-erbA* was generated by excising the coding sequence of the cloned *gag-v-erbA* gene²⁴, followed by appropriate modification of the 5' and 3' ends and insertion between the *Kpn*I and *Bam*H1 sites of the vector pRS-hGR_{NX}³¹. Synthetic oligonucleotides encoding a palindromic response element (TRE; TCAGGTCATGACCTGA) (ref 22) flanked by *Hind*III adaptor sequences were inserted into the unique *Hind*III cloning site in pBL-CAT²³. Plasmids containing one or multiple copies of the TRE were identified by restriction enzyme mapping and sequence analysis.

plementary DNA or the *v-erbA* oncogene under the transcriptional control of the Rous sarcoma virus (RSV) long terminal repeat were co-transfected with one of the reporter plasmids into CV1 cells, which lack significant levels of endogenous thyroid-hormone receptor (TR).

Transfection of *tk-TRE_p-CAT*, containing a palindromic response element, or the parental vector *tk-CAT* resulted in a high basal level of CAT activity that was only marginally stimulated by the addition of thyroid hormone (Fig. 2; Table 1). In contrast, co-transfection with the TR expression vector RS-rTR α resulted in marked effects on *tk-TRE_p-CAT* expression. In the absence of thyroid hormone, we observed an 80% decrease in basal CAT activity (referred to as 'low basal-level activity'), indicating that TR expression provokes a ligand-independent inhibitory effect on transcription (Fig. 2; Table 1). Addition of T₃ to a final concentration of 100 nM resulted in a 20-fold stimulation of *tk-TRE_p-CAT*. This corresponds to a 3- to 5-fold stimulation over the 'high basal-level activity' in the absence of TR expression (Fig. 2; Table 1). Reporter constructs containing multiple copies of the palindromic response element (*TRE_{p2}-CAT*, *TRE_{p3}-CAT*) reveal a strong synergistic effect on both repression and activation, resulting in a 100- to 200-fold induction over the repressed level (Table 1). This regulatory function of the rTR α is not restricted to the *TRE_p*; as shown in Fig. 2, the TRE from the rat growth-hormone gene (*TRE_{GH3}*) is also able to sustain hormone-independent and dependent transcriptional responses.

This functional assay enables a direct determination of the putative transcriptional activity of the *v-erbA* oncogene product. As *v-erbA* has lost its ability to bind thyroid hormone but retains an intact DNA-binding domain, it seems logical that it would function as a constitutively active TR. Unexpectedly, co-transfection of *v-erbA* with either reporter plasmid did not stimulate the transcription, but rather resembled the negative regulatory effects of rTR α in the absence of hormone. Thus, in cells expressing *v-erbA*, CAT activity was reduced by 80% from the high basal level, and could not be relieved by the addition of T₃ (Fig. 2).

Chimaeric proteins

To identify the contribution of the different mutations in *v-erbA* to the altered properties of its protein product and to further dissect the processes of activation and repression, chimaeric receptors between *v-erbA*^{4,24} and the rat TR α ²⁰ were constructed. Between amino acids 154 and 410, *v-erbA* differs from rTR α in 26 amino acids and a deletion of 9 amino acids close to the C-terminus (see legend to Fig. 1). Substitution of this region between rTR α and *v-erbA* gives rise to the hybrid TR(154)erbA (Fig. 3a), the properties of which are virtually identical to the

v-erbA homologue (Fig. 3b). Thus, the mutations in the DNA-binding domain and flanking amino acids are not crucial to the *v-erbA* phenotype. Substitution of an internal region of the ligand-binding domain, amino acids 154–316, creates a T₃-responsive hybrid (TR(154/316)erbA) which functions like the natural receptor (Fig. 3a, b). In contrast, replacement of the C-terminal 93 amino acids of rTR α with the corresponding sequence of *v-erbA*, containing the 9-amino-acid deletion and additional 11-amino-acid differences, yielded the hybrid protein TR(317)erbA, with suppressor properties identical to the viral oncogene product (Fig. 3a, b).

DNA binding

These data suggest that the ability of rTR α to inhibit CAT expression may be a direct consequence of binding to its response element in the unliganded state. To test this hypothesis,

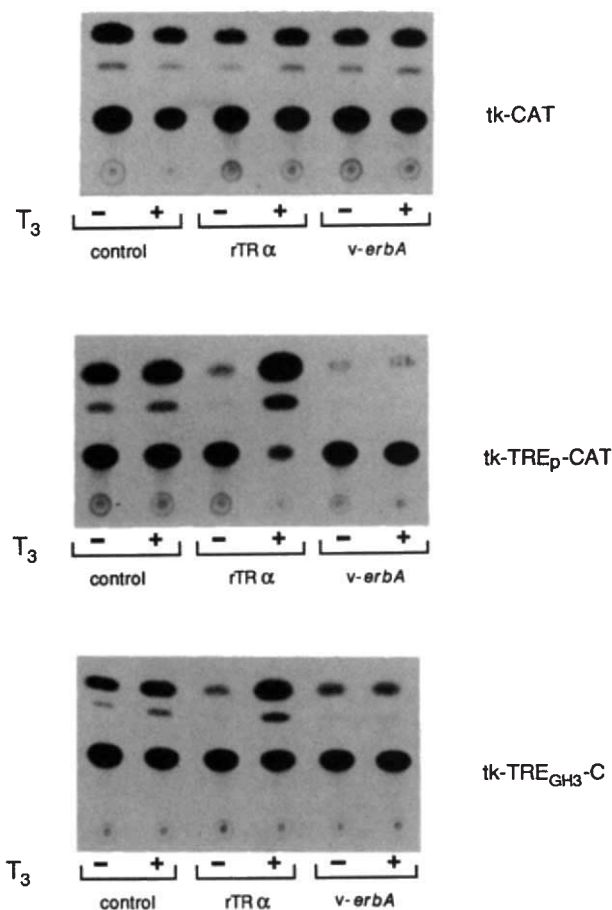


FIG. 2 Reporter gene regulation. CV1 cells were transfected with the reporter construct *tk-CAT*, *tk-TRE_p-CAT* or *tk-TRE_{GH3}-CAT*, the respective expression plasmid (RS-rTR α or RS-*v-erbA*) and the internal reference plasmid RSV- β GAL. In the control experiments, a construct carrying the rTR α coding sequences in reverse orientation (RS-3'-5') was used. Thyroid hormone (T₃) was added as indicated. Results shown are corrected for β -galactosidase expression from RSV- β GAL. Acetylated and non-acetylated forms of [¹⁴C]chloramphenicol were separated by thin-layer chromatography and the results of typical experiments are shown.

METHODS. CV1 cells were maintained in DME medium supplemented with 5% calf bovine serum and transfected at 30–50% confluency with a total of 20 μ g DNA by using the calcium phosphate co-precipitation technique. Expression (5 μ g) and reporter (2.5 μ g) plasmid DNA, together with 2.5 μ g RSV- β GAL as an internal control for transfection efficiency, were cotransfected per 10-cm dish. Transfected cells were grown in 10% resin-treated fetal calf serum³² minus or plus 10⁻⁷ M 3,5,3'-triiodothyronine (T₃). Cells were collected 40 h after the addition of T₃ and β -galactosidase and CAT-assays performed as described^{33,34}. The plasmid *tk-TRE_{GH3}-CAT* contains the 29-base pair (bp) TRE from the rat growth hormone gene in three copies (G293TK in ref. 21).

TABLE 1 Activity of rTR α is enhanced by the presence of multiple response elements

	Control		rTR α		Fold induction*
	—	T ₃	—	T ₃	
<i>tk-CAT</i>	1.0	2.0	0.5	1.4	3
<i>tk-TRE_p-CAT</i>	1.0	1.5	0.2	3.8	19
<i>tk-TRE_{p2}-CAT</i>	1.0	2.6	0.07	12.5	180
<i>tk-TRE_{p3}-CAT</i>	1.0	2.4	0.08	11.0	140

The results shown are the average of three independent transfection experiments. Reporter plasmids containing one or multiple copies of the palindromic TRE_p were identified by restriction enzyme mapping and sequence analysis. The transfection of CV1 cells, β -galactosidase and CAT-assays were performed as described in Fig. 2. Acetylated and non-acetylated forms of [¹⁴C]-chloramphenicol were separated by thin-layer chromatography and quantitated by liquid-scintillation counting. 'High basal-level activity' was set to 1.0 in the individual reporter constructs and refers to a 5–15% chloramphenicol conversion.

* Calculated from the 'low basal level activity'.

COS cells were transiently transfected with the expression plasmids, containing the simian virus 40 (SV40) origin of replication to ensure high intracellular levels of receptor proteins. Whole-cell extracts of cells expressing rTR α were incubated with the ³²P-labelled TRE_p, followed by electrophoresis in a non-denaturing gel. A protein-DNA complex migrating more slowly than the free DNA was observed, regardless of whether the cells were cultured in the absence or presence of T₃ (Fig. 4a). The specific complex could be displaced by unlabelled TRE_p but not by non-specific DNA. Similar hormone-independent receptor-DNA interaction could be demonstrated *in vitro*. T₃ had no effect on the appearance of the retarded complex when it was added to extracts of transfected COS cells which were not exposed to the hormone *in vivo* (Fig. 4a). Hormone-independent formation of retarded complexes was also observed with extracts from cells transfected with RS-*v-erbA* (Fig. 4b). The difference observed in the amount of labelled DNA displaced by *v-erbA* and rTR α might reflect a higher affinity of *v-erbA* for this response element or a higher amount of *v-erbA* protein in the cell extracts. However, as shown in Fig. 3b, correcting the mutations in the *v-erbA* DNA-binding domain towards wild-type rTR α does not apparently weaken the suppression and the chimera TR(154)*erbA* forms the expected retarded complex *in vitro* (Fig. 4b). Extracts of cells transfected with RS-3'-5', containing the rTR α sequence in anti-sense orientation, or i95TR, a derivative of rTR α with an in-frame insertion at amino-acid position 95 in the DNA-binding domain, however, showed no DNA-binding activity.

Cotransfection and competition

In AEV-infected erythroid cells, therefore, *v-erbA* might act to compete and thus interfere with the function of the endogenous TR/*c-erbA*. We have performed co-transfection studies to examine the effect of *v-erbA* on rTR α function in CV1 cells. As shown in Fig. 5a, a 10-fold molar excess of RS-*v-erbA* reduces by 90% the thyroid-hormone dependent induction of the reporter-gene transcription by rTR α . The receptor hybrids TR(154)*erbA* and TR(317)*erbA* also exhibited *v-erbA*-like activities, provoking a virtually complete repression of the T₃ and rTR α induced stimulation of the reporter gene (Fig. 5a). This again demonstrates that the mutations in the DNA-binding domain of *v-erbA* are not crucial in this assay (Fig. 5a). But the activity is dependent on an intact DNA-binding region as the DNA-binding domain mutant i95(154)*erbA* fails to compete (Fig. 5a). Similar competition of T₃ induction by *v-erbA* and the chimaeric constructs was observed with the TRE_p placed in the context of the mouse mammary tumour virus promoter (data not shown). One prediction of these results is that an inverse relationship exists between the activity of the TR and the concentration of the competitor product. To examine this, varying molar ratios of TR(154)*erbA* and the rTR α expression vector were co-transfected and hormone responsiveness was assessed. As expected, increasing quantities of TR(154)*erbA* lead to decreasing activity of the rTR α (Fig. 5b). In this assay, even small amounts of TR(154)*erbA* are potent, with a 3:1 plasmid ratio completely blunting the hormone-induced response.

Discussion

The results of this study demonstrate that the *v-erbA* oncogene product is (1) able to selectively bind to specific DNA sequences *in vitro*, (2) can act as a constitutive repressor of thyroid-hormone-responsive genes, and (3) when co-expressed with its progenitor, the *c-erbA*/thyroid-hormone-receptor gene, will virtually eliminate an otherwise potent transcriptional response to thyroid hormone. Our data support the idea that the contribution of *v-erbA* to erythroblast transformation is probably the result of blocking thyroid-hormone-induced differentiation. According to this model, *v-erbA* may be a member of a new class of growth regulator, the dominant negative oncogene, considered on theoretical grounds by Herskowitz²⁵.

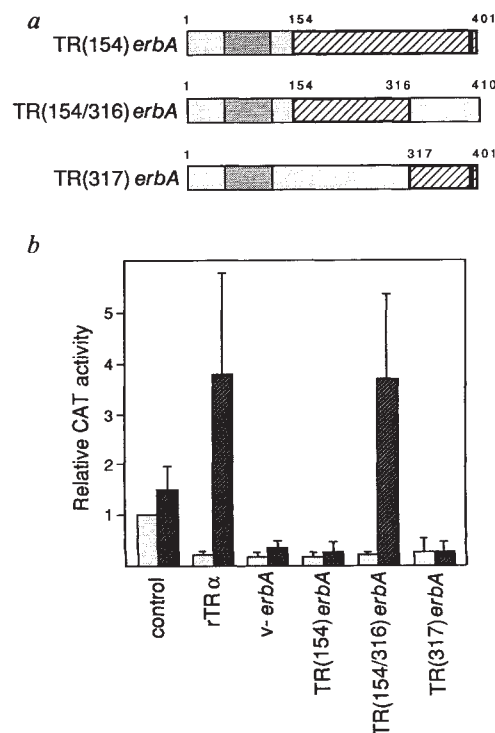


FIG. 3 Structure and activity of rat TR α /*v-erbA* chimaeric proteins. *a*, Schematic representation of the structure of the rat TR α /*v-erbA* chimaeric proteins. Numbers on top indicate amino-acid positions. The black bar indicates the deletion of 9 amino acids in *v-erbA*, resulting in fusion proteins of 401 amino acids compared with the 410 amino acid of the protein with intact C-terminus. *b*, Positive and negative regulation of CAT activity from *tk*-TRE_p-CAT using the indicated expression vectors. Shown in the histogram are the average values of 2-6 independent transfection experiments. Stippled bars, no hormone, striped bars, +100 nM T₃.

METHODS. Hybrid genes were constructed using restriction sites common to both rTR α ²⁰ and *v-erbA* genes^{4,24}. TR(Δ154/317) was created by deleting the *Pst*I fragment in the ligand-binding domain of rTR α . Replacement of the rTR α C-terminus by a *Pst*I-*Xba*I fragment from a *v-erbA*-*neo* construct⁸ generated TR(Δ154/317)*erbA*. The plasmids TR(317)*erbA*, TR(154)*erbA* and TR(154/317)*erbA* were generated by reinserting the *Pst*I fragments from either rTR α or *v-erbA* into the unique *Pst*I site of either plasmid. All modifications were performed on a subcloned fragment from the ligand binding domain of rTR α and hybrid expression constructs were generated by replacing the *Xba*I fragment of RS-rTR α with the corresponding chimaeric fragments. Transfections of CV1 cells, β -galactosidase and CAT assays were performed as described above.

Although it is generally assumed that the ability of a hormone receptor to bind DNA is ligand-dependent¹⁰, our data and previous observations²⁶ challenge this convention. The down-regulation of transcription from the responsive promoter is presumably a consequence of the ability of the thyroid hormone receptor to recognize and bind to its cognate response element in the absence of hormone. Two pieces of evidence support this proposal. First, an intact DNA-binding domain is necessary for both the gel-retardation experiments and the observed transcriptional effects. Second, in the absence of the response element no significant repression is observed, whereas a tandem copy of the TRE potentiates both positive and negative transcriptional effects. Although positive synergism has been observed by glucocorticoid and oestrogen receptors and their respective response elements^{27,28} the negative synergism observed here is apparently without precedent.

These results suggest that the steroid- and thyroid-hormone receptors have adopted different strategies to control DNA binding. In the case of rTR α , the primary, and possibly the only, effect of the hormone is to trigger a conformational change in

the DNA-bound receptor that relieves the repressor activity and reveals or induces the activation function. In this context, v-erbA acts as a constitutive repressor of gene transcription because it fails to bind hormone and the activating function cannot be revealed. The effectiveness of v-erbA in blocking TR function is puzzling and may in part be a consequence of higher protein levels and/or altered DNA-binding properties. In the absence of any information on the relative binding affinities of v-erbA and rTR α , or on their concentrations in transfected cells, only limited inferences on their interactions can be drawn. Because an intact DNA-binding domain is essential for v-erbA function,

the competition or antagonism apparently occurs at the level of the response element. This could either block the binding of the TR to the template or its hormone-induced activation. Whether repression and activation are mediated by identical or distinct structural domains is unknown and additional experiments analysing the requirement of specific sequences in the C-terminus for these functions are in progress. It is noteworthy that alternatively spliced versions of the thyroid-hormone receptor might also inhibit rTR α functions²⁹. But in these experiments no repression of basal promoter activity by the non-hormone-binding forms has been observed.

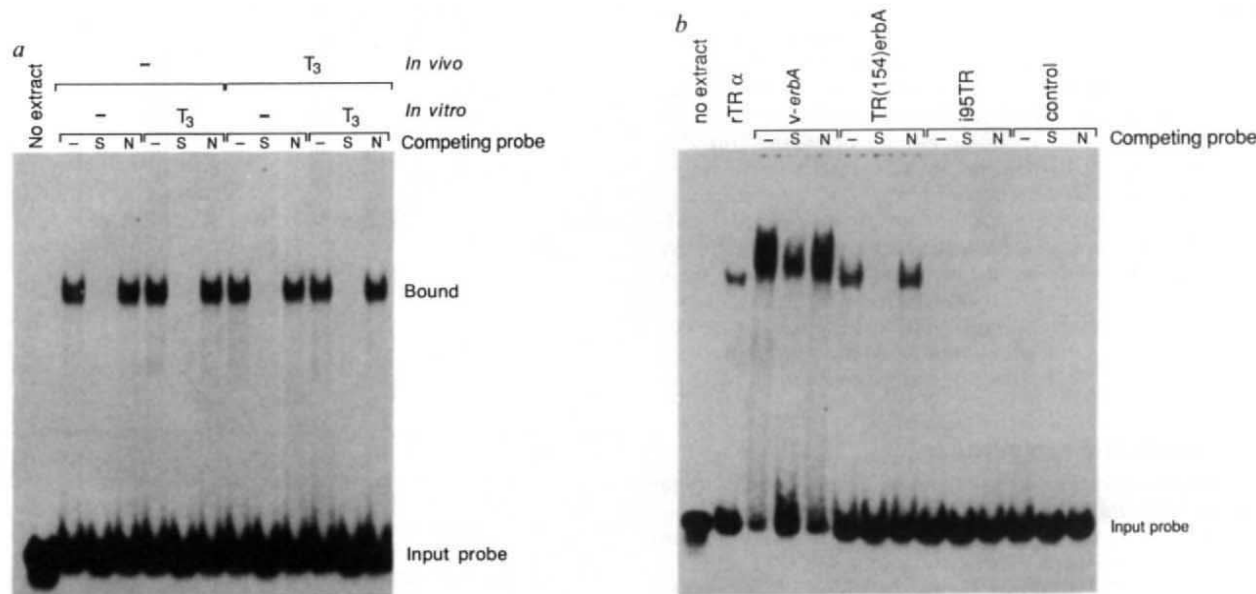


FIG. 4 DNA binding by rTR α and v-erbA proteins *in vitro*. *a*, rTR α binds DNA *in vitro* in the absence of ligand. Extracts of COS cells transfected with RS-rTR α were prepared and incubated with a ³²P-labelled oligonucleotide encoding TRE_p. Protein-bound and free DNA were separated by electrophoresis through non-denaturing gels and visualized by autoradiography. 'In vivo' indicates whether cells were grown in the absence (–) or presence of T₃ before collecting. 'In vitro' indicates whether extracts were incubated in the absence (–) or presence of T₃ in the DNA-binding reaction. Competing probe indicates the addition of specific (S) or non-specific (N) unlabelled competitor DNA at 15-fold molar excess. S, TRE_p; N, *cis*-active element from adenovirus long terminal repeat which does not compete for binding to T₃ receptor²². *b*, Binding of v-erbA to TRE_p *in vitro*. Extracts from COS cells transfected with the indicated expression plasmids and grown in the absence of hormone were used in DNA-binding assays. Expression vector with rTR α in the anti-sense orientation was transfected as a negative control. Competitor DNAs are the same as in *a*.

METHODS. COS cells were grown in DMEM with 5% T₃ free bovine serum and transfected using DEAE-dextran³⁵. After 36 h, cells were collected and extracts prepared as described³⁶ except that the buffer was 20 mM HEPES, pH 7.8, 0.4 M KCl, 2 mM dithiothreitol and 20% glycerol. DNA-binding reactions and gel electrophoresis were performed essentially as described²². Aliquots containing 6–10 μ g total protein were diluted so that the final concentration of KCl was 80 mM, then incubated with 2 μ g poly d(I·C) for 20 min at room temperature. At this time, 25–50 fmols of a ³²P-labelled oligonucleotide encoding the palindromic TRE was added. The reaction mixture was incubated at 22 °C for 30 min and then loaded on a 5% polyacrylamide gel containing 50 mM HEPES, pH 7.8. Competitor DNAs were added before the addition of the labelled oligonucleotide. The DNA-binding mutant, i95TR, was constructed by partial digestion of rTR α with *PvuII* and adding a *Bam*HI linker (12-mer) to restore the open reading frame. The position of the linker was verified by restriction-enzyme mapping and sequence analysis.

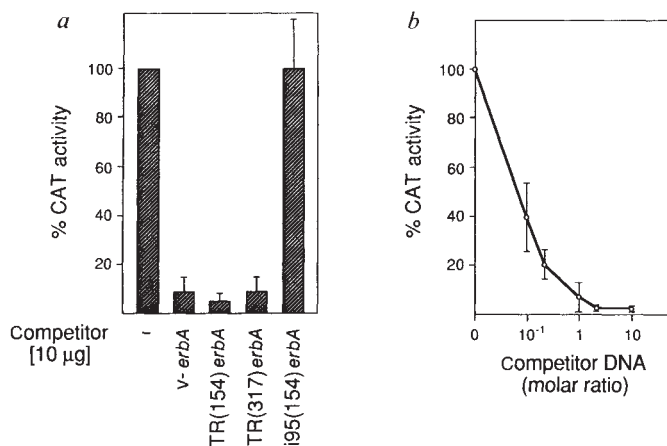


FIG. 5 Competition of T₃ induction. *a*, The reporter gene tk-TRE_p-CAT (0.5 μ g) was co-transfected into CV1 cells with 1 μ g rTR α expression vector and the internal control plasmid RSV- β GAL. In addition, a 10-fold excess (10 μ g) of the indicated expression plasmids was cotransfected with rTR α . In (–), 10 μ g RS-3'-5' was used. The average values of 2–6 independent transfection experiments are shown in the histogram. All experiments were performed in the presence of 100 nM T₃. *b*, The reporter gene tk-TRE_p-CAT (0.5 μ g) was cotransfected into CV1 cells with 1 μ g expression vector RS-rTR α and increasing quantities of the non-hormone-binding competitor TR(154)erbA. The amount of RSV-promoter was held constant in all transfections by the addition of RS-3'-5'. Shown are the average values of three independent transfection experiments. Cells were grown in the presence of 100 nM T₃.

METHODS. The DNA-binding mutant i95(154)erbA was constructed by partial digestion of TR(154)erbA with *PvuII* and adding a *Bam*HI linker (12-mer) to restore the open reading frame. The position of the linker was verified by restriction-enzyme mapping and sequence analysis. Transfection of CV1 cells, β -galactosidase and CAT assays were as described.

The 'tumour suppressor' or 'anti-oncogene'² bears an intriguing relationship to the erbA phenotype described here. The inactivation or loss of both copies of these genes has been genetically implicated in a number of human tumours^{2,30}. Thus, it is the absence of the product that leads to transformation in some cell types and in these cases tumour development requires at least two genetic changes. The experiments in this study

suggest that v-erbA contributes to cellular transformation by inhibiting the function of its normal endogenous counterpart. We propose that in some cases, loss of function resulting from a single mutation would be sufficient as a causal event in tumorigenesis for some recessive oncogenes. Therefore, the dominant negative phenotype may be a prototype of a new and more general mechanism of cellular transformation. □

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Structural parts involved in activation and inactivation of the sodium channel

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Structure–function relationships of the sodium channel expressed in *Xenopus* oocytes have been investigated by the combined use of site-directed mutagenesis and patch-clamp recording. This study provides evidence that the positive charges in segment S4 are involved in the voltage-sensing mechanism for activation of the channel and that the region between repeats III and IV is important for its inactivation.

THE voltage-gated sodium channel is essential for the generation of action potentials in many excitable cells^{1,2}. Voltage-gated ionic channels must have charged structures that can move within the membrane and thus sense the membrane electric field. These movements produce a measurable current called the gating current^{3,4}, but the localization and identification of these structures within a channel protein are uncertain. The primary structures of the *Electrophorus* electroplax sodium channel⁵ and of three distinct sodium channels from rat brain^{6,7} have been elucidated by cloning and sequence analysis of the complementary DNAs. The partial amino-acid sequence of the sodium channel from *Drosophila* has also been deduced from the genomic DNA sequence⁸. The sodium channel is composed of

about 2,000 amino-acid residues and contains four homologous internal repeats, each of which has six putative transmembrane segments. One of these segments, S4, contains several arginine or lysine residues at every third position with mostly nonpolar residues intervening between the basic residues. It has therefore been proposed that the positive charges in segment S4 serve as voltage sensor^{5,6}. This hypothesis is supported by the fact that the unique structural features of segment S4 are shared by the slow calcium channel from rabbit skeletal muscle^{9,10} and by potassium channels from *Drosophila*^{11–13} and mammalian brain^{14,15}. Functional sodium channels are expressed in *Xenopus* oocytes by microinjection of messenger RNAs derived from rat brain sodium channel cDNAs^{16–19}. The sodium channels expressed from the cDNAs closely resemble those from native membranes in their properties, including the sensitivity to tetrodotoxin and saxitoxin, the rate at which the current turns on in response to a depolarizing voltage step (activation), the decay of the current during a maintained depolarization (inactivation) and the single-channel conductance.

To identify functional regions of the sodium channel, we have now investigated the effects of site-directed mutations of rat sodium channel II on its functional properties. First, to test the proposed role of segment S4 as voltage sensor, we have introduced point mutations into the S4 region so that positively charged amino-acid residues are replaced by neutral or negatively charged residues. A reduction in the net positive charge in segment S4 of repeat I leads to a decrease in the steepness of the potential dependence of activation, which provides experimental evidence for the direct involvement of the positive

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charges in this segment in the voltage-sensing device for activation. Second, to localize a region involved in inactivation, we have examined the effects of deletion or cleavage in putative cytoplasmic portions of the channel protein. Some of these mutants form functional sodium channels, two of which are characterized by inactivation rates much lower than that of the wild-type channel.

Construction of mutants

To facilitate the construction of mutant cDNAs, we prepared a recombinant plasmid, pRII-2A, which is a derivative of the plasmid pRII-2 (ref. 16) and carries the entire protein-coding sequence of the rat sodium channel II cDNA linked with the bacteriophage SP6 promoter. In pRII-2A, several unique restriction endonuclease sites have been introduced without altering the amino-acid sequence of sodium channel II (Fig. 1a). We confirmed that the messengerRNA derived from pRII-2A, when injected into *Xenopus* oocytes, yielded a sodium channel with functional properties indistinguishable from those of the sodium channel derived from pRII-2 (refs 16, 17). In the present investigation, pRII-2A was used to produce the mRNA specific for the wild-type sodium channel, and all plasmids carrying mutant

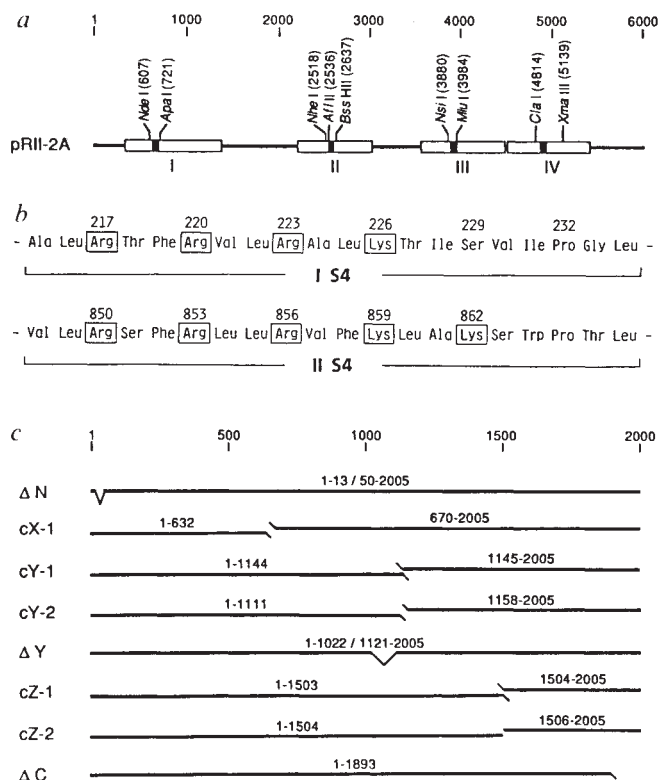
cDNAs were constructed from pRII-2A. mRNAs synthesized by transcription *in vitro* of the cDNAs were injected into *Xenopus* oocytes and the resulting sodium channels were examined primarily by recording macroscopic sodium currents through cell-attached membrane patches of large diameter¹⁷. Current records were analysed according to the Hodgkin-Huxley (HH) equations¹ for the voltage dependence of the steady-state activation (m_{∞}) and inactivation (h_{∞}) parameters and of the respective time constants (τ_m and τ_h). The HH scheme, especially in that it treats m and h as independent parameters, does not provide a faithful interpretation of the actual set of structural transitions underlying the operation of sodium channels^{20,21}. In the absence of any generally accepted more realistic scheme, however, the HH equations remain the most useful empirical framework for a fairly accurate description of the electrophysiological data with a minimum number of parameters.

Effects on activation

It is assumed that many of the positive charges in the putative voltage sensor segment S4 form ion pairs with negative charges in other transmembrane segments. The breakage of these ion

FIG. 1 Strategies for the construction of rat sodium channel II cDNA mutants. a, Restriction map of the recombinant plasmid pRII-2A used for site-directed mutagenesis. Only the protein-coding region is shown and nucleotide numbers⁶ are given above the diagram. The coding regions for the four internal repeats (I–IV) are indicated by open boxes, and those for segment S4 in each repeat by filled boxes. The restriction endonuclease sites introduced are shown and identified by numbers (in parentheses) indicating the 5'-terminal nucleotide generated by cleavage. b, Amino-acid sequences⁶ of segment S4 in repeat I (IS4) and repeat II (IIS4) of wild-type rat sodium channel II. The termini of the segments are tentatively assigned. Positively charged residues are boxed with solid lines and the numbers⁶ of the relevant residues are given. c, Structures of deletion mutants and their combinations. The regions of rat sodium channel II carried by the individual mutants are shown by horizontal lines with the numbers of the constituent amino-acid residues; oblique lines at the termini of some constructs indicate the presence of short additional sequences (see text and Methods). V-shaped lines indicate internal deletions. Amino-acid numbers⁶ are given above the diagram.

METHODS. The rat sodium channel II cDNA sequence in the plasmid pRII-2 (ref. 16) was modified by introducing unique restriction endonuclease sites into both sides of the coding region for each segment S4 to yield pRII-2A; the *Xba*I(541)/*Apa*I(721), *Dde*I(2498)/*Sph*I(2701), *Sau*3A1(3840)/*Ban*I(3995), *Fok*I(4793)/*Hha*I(4978) and *Sau*3A1(5112)/*Hpa*II(5142) segments were replaced by synthetic DNAs. pRII-2A differs from pRII-2 as follows (the substituted nucleotides with residue numbers⁶ are given): A, 606, 2,520, 2,538, 2,539, 3,879, 4,812; G, 2,517, 2,540, 2,640, 3,987, 3,990, 4,815, 5,139; C, 2,518, 2,535, 2,637; T, 2,541, 3,988. Point mutations were introduced by replacing the *Nde*II(607)/*Apa*I(721) and/or the *Afl*III(2536)/*Bss*HI(2637) segment of pRII-2A with synthetic DNAs carrying the respective mutations; some silent mutations were also introduced to generate restriction endonuclease sites used for identification of the plasmids constructed. The mutant plasmids differ from pRII-2A as follows (the substituted nucleotides with residue numbers⁶ are given and the plasmids carrying mutated cDNAs are named after the mutant specification). pAM(R217Q): G, 648, 651, 699; A, 650; C, 696. pAM(R220Q): CAG, 658–660; C, 696; G, 699. pAM(R223Q): C, 667, 696; A, 668; G, 699. pAM(K226Q): C, 673, 676; G, 678. pAM(K226E): G, 676, 678, 699; C, 696. pAM(K226D): C, 673, 675; G, 676; T, 678. pAM(K226R): C, 672; A, 675; G, 677. pAM(S229R): G, 669; C, 672, 673; AGA, 685–687. pAM(R217Q-R220Q): G, 648, 651, 699; A, 650; CAG, 658–660; C, 696. pAM(R217Q-R223Q): G, 648, 651, 699; A, 650, 668; C, 667, 696. pAM(R217Q-K226Q): G, 648, 651, 678; A, 650; C, 673, 676. pAM(R220Q-R223Q): CAG, 658–660; C, 667, 696; A, 668; G, 699. pAM(R223Q-K226Q): CAG, 658–660; C, 673, 676; G, 678. pAM(K226R-S229R): C, 672; A, 675; G, 677; AGA, 685–687. pAM(S229K-P232R): C, 673, 693, 696; T, 675; G, 678, 695; AAA, 685–687. pAM(R217Q-R220Q-R223Q): G, 648, 651, 699; A, 650, 668; CAG, 658–660; C, 667, 696. pAM(R217Q-R220Q-K226Q): G, 648, 651, 678; A, 650; CAG, 658–660; C, 673, 676. pAM(K862Q): T, 2,583; C, 2,584. pAM(K859Q-K862Q): C, 2,575, 2,578, 2,584. pAM(K226Q-K859Q-K862Q): C, 673, 676, 2,575, 2,578, 2,584; G, 678. Deletion mutants were constructed by removing a cDNA segment



from pRII-2A using restriction endonucleases, exonuclease III and/or mung bean nuclease. For the construction of pADI/II/III-2 and pADIV-2, synthetic oligonucleotides were also used to introduce a termination or an initiation codon. Sodium channel mutants derived from the individual plasmids or their combinations are as follows (the numbers of the amino-acid residues encoded and the additional C-terminal sequence resulting from the strategy used, if any, are given in parentheses): ΔN: pADN (1–13/50–2,005). cX-1: pADI-2 (1–632 plus HQGK) plus pADI/II/IV-2 (1–6/670–2,005). cX-2: pADI-1 (1–481 plus DQGK) plus pADI/II/IV-1 (1–6/583–2,005). cX-3: pADI-1 plus pADI/II/IV-2. cY-1: pADI/II-1 (1–1,144/2,000–2,005) plus pADI/III/IV-1 (1–6/1,145–2,005). cY-2: pADI/II-2 (1–1,111 plus YQGK) plus pADI/III/IV-2 (1–6/1,158–2,005). ΔY: pADY (1–1,022/1,121–2,005). cZ-1: pADI/II/III-1 (1–1,503/2,000–2,005) plus pADI/IV-1 (1–8/1,504–2,005). cZ-2: pADI/II/III-2 (1–1,504) plus pAD IV-2 (initiating methionine plus 1,506–2,005). ΔC: pADC (1–1,893 plus DQGK). All the constructs were confirmed by sequencing³⁰ the portions encompassing the synthetic DNA and ligation sites and by restriction endonuclease analysis.

pairs and the displacement of segment S4 within the transmembrane electric field may be the origin of the charge movement that accompanies the opening and closing of the sodium channel pore and confers voltage dependence on this gating process. If this is the case, replacement of a positively charged residue in segment S4 by a neutral or a negatively charged residue would be expected to alter the gating properties of the sodium channel. We investigated several mutants of this type in which mostly the S4 region of repeat I (Fig. 1b) was altered. Mutagenesis was focused on this region because it is the S4 segment which has the least number of positively charged residues and thus the largest effects of charge modifications would be anticipated. A few mutations in segment S4 of repeat II (Fig. 1b) were also included.

No appreciable expression of functional sodium channels in *Xenopus* oocytes was observed for any of the mutants that involved neutralization of more than three positive charges in segment S4 of repeat I and/or repeat II. All the single-, double- and triple-site mutations tested, however, yielded measurable expression of sodium channels. Mutations are indicated by the amino-acid residues (in one-letter code) in the wild-type and the mutant preceding and following the number⁶ of the altered residue, respectively.

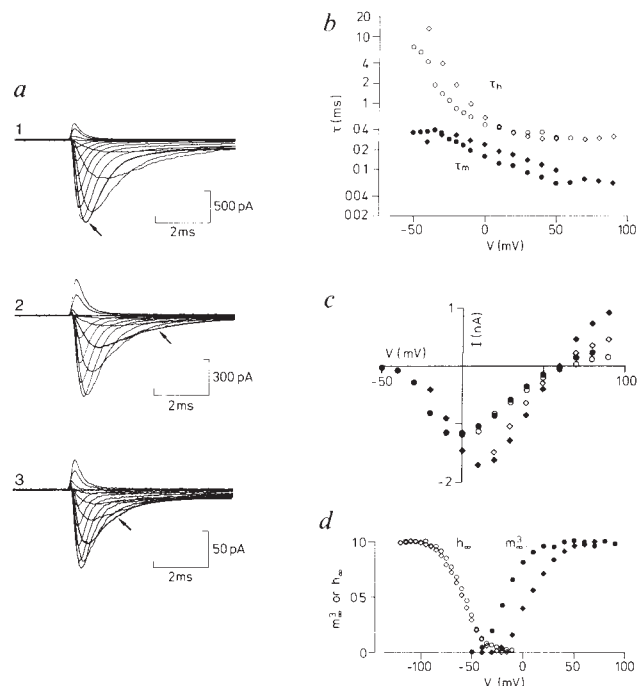
Figure 2a exemplifies current records for the wild-type sodium channel (1) and two mutants, K226Q (2) and R217Q·K226Q (3). The current records were fitted to m^3h kinetics using a least-squares method to yield time constants for activation (τ_m) and inactivation (τ_h); examples of least-squares fits are shown by the smooth lines superimposed on the responses to -20 mV (indicated by arrows). The non-linear voltage dependence of instantaneous currents, assessed by tail current analysis²², was

FIG. 2 a, Current responses to depolarizations in oocytes implanted with the wild-type (1) or with the mutant sodium channels K226Q (2) and R217Q·K226Q (3). *Xenopus* oocytes were injected with the wild type (0.20 $\mu\text{g } \mu\text{l}^{-1}$), K226Q (0.33 $\mu\text{g } \mu\text{l}^{-1}$) or R217Q·K226Q (0.33 $\mu\text{g } \mu\text{l}^{-1}$) mRNA. The responses were evoked by depolarizations ranging -60 – $+70$ mV in 10-mV steps from a holding potential of -120 mV and they were recorded from cell-attached macro-patches^{17,27}. Temperature: 15°C . The data shown are the averages of 4 (1, 2) or 16 (3) individual traces. The current signals have been corrected for leakage and capacitive transients. Smooth lines superimposed on the -20 -mV responses (arrows) show examples of least-squares fits according to HH-like kinetics as described below. b, Example of the voltage dependence of the time constants of activation (τ_m , filled symbols) and fast inactivation (τ_h , open symbols). Circles: wild-type; diamonds: K226Q. c, Example of the voltage dependence of peak currents (I_p , closed symbols) and instantaneous tail currents following a conditioning depolarization to $+30$ mV (I_t , open symbols). Circles: wild-type; diamonds: K226Q. d, Example of steady-state activation (m_∞^3 , filled symbols) and inactivation (h_∞ , open symbols) curves for wild-type (circles) and K226Q (diamonds) sodium channels.

METHODS. Records in a were fitted to m^3h kinetics plus a delay, δt , which decreased with depolarization and, for a holding potential of -120 mV, ranged from 0–110 μs (ref. 31). The fitting of each current response, $I(t)$, proceeded as follows. The decay phase of $I(t)$ was least-squares fitted with a double exponential function, $I_f(t)$, comprising a major fast component of inactivation with time constant τ_h and a slower component with a relative amplitude of less than 10% which is not further discussed. In a short interval around the peak, $I(t)$ was least-squares fitted with a third-order polynomial to obtain the best guess of the peak current, I_p , and of the time to the peak, t_p . The activation time constant, τ_m , and the delay, δt , were obtained from the least-squares fit during the rising phase of $I(t)/I_f(t)$ to the function $(1 - \exp(-(t - \delta t)/\tau_m))^3$ up to $1.5 t_p$. Assuming the same delay for m and h , $I_f(\delta t)$ was taken as the apparent steady-state current in the absence of inactivation, I' . Instantaneous tail currents, I_t , were obtained from separate protocols of stimulation comprising a fixed conditioning prepulse to $+30$ mV with duration $\sim t_p$ followed by a step to a variable tail potential. As discussed in ref. 22, division by I_t unfolds from I' the non-linear voltage dependence of the open-channel current, so that the asymptotic open-channel probability in the absence of inactivation, m_∞^3 , was assumed to be proportional to the ratio I'/I_t . The latter quantity was least-squares fitted, assuming a voltage-dependence of m_∞ of the type:

used to translate the apparent steady-state current in the absence of inactivation into an apparent asymptotic open-channel probability (m_∞^3). The voltage dependence of steady-state inactivation (h_∞) was obtained according to standard protocols¹⁷. Figures 2b, c and d show the voltage dependence of τ_m and τ_h , of peak currents and instantaneous tail currents and of m_∞^3 and h_∞ , respectively, for the wild-type and the mutant K226Q.

The functional properties of the mutants studied are summarized in Table 1, which gives, among others, the potentials at which the HH parameters for activation and inactivation are half-maximal ($V_{1/2}^m$ and $V_{1/2}^h$), the valence of the apparent single-gate charge for activation (z_m) and the steepness of the potential dependence of inactivation (a_h). A decrease in z_m and thus a decrease in the slope of the relation of steady-state activation (m_∞) versus membrane potential is expected to result from a reduction in the net charge in those elements involved in the potential-dependent gating process. This is indeed the case for some mutant sodium channels, as exemplified in Fig. 3a. Replacement of one arginine or lysine residue in segment S4 of repeat I by a glutamine residue causes a significant reduction in z_m (R220Q, R223Q and K226Q), except for the mutation at position 217 (R217Q) (Table 1). However, double mutations combining R217Q with neutralization of positive charge at another site (R217Q·R220Q, R217Q·R223Q and R217Q·K226Q) intensify the effects of the latter mutations. The mutations K226E and K226D, which reduce the net positive charge at position 226 by two units, cause a stronger reduction in z_m than does the mutation K226Q. By contrast, the mutation K226R, which does not alter the positive charge at position 226, does not affect the z_m value. In Fig. 3b, the valence of the apparent single-gate charge, z_m , is plotted as a function of the decrease in the total



$$m_\infty(V) = 1 / \left(1 + \exp \left(\frac{z_m e_0 (V - V_{1/2}^m)}{kT} \right) \right)$$

where e_0 is the electron charge (-1.60×10^{-19} coulomb), k is Boltzmann's constant and T is the absolute temperature. h_∞ plots were obtained from standard protocols¹⁷ of voltage-clamp responses to test depolarizations eliciting maximal inward currents following 100 ms prepulses of variable amplitude. The data were least-squares fitted by a function of the type:

$$h_\infty(V) = 1 / \left(1 + \exp \left(\frac{V - V_{1/2}^h}{a_h} \right) \right)$$

number of net positive charges in segment S4 of repeat I. A roughly inverse relationship is observed between the z_m value and the decrease in total net positive charges. This finding provides experimental evidence that the positive charges in segment S4 of repeat I are directly involved in the voltage-sensing mechanism for activation. Although z_m is merely an empirical parameter whose absolute value is dictated by the use of the HH scheme, it should be stressed that the above effects are qualitatively model-independent because they are ultimately related to changes in the steepness of the voltage dependence of activation.

Extension of the S4 motif towards the C-terminus by substituting positively charged residues for neutral residues (S229R, K226R·S229R and S229K·P232R) does not increase the z_m value (Table 1). This suggests either that the side chains at positions 229 and 232 are not located within the membrane and are thus unable to sense the transmembrane field, or that they do not move along the field with gating. Neutralization of positive charges in segment S4 of repeat II at positions 859 and 862 exerts little effect on z_m (Table 1). No definite conclusion, however, can be drawn as to the role of segment S4 of repeat II because we have not systematically tested mutations of all the positively charged residues in this segment.

Some mutations that neutralize positive charges in segment

S4 of repeat I cause shifts in the potential dependence of activation, as exemplified in Fig. 3c. In particular, the mutation K226Q, which involves neutralization of the last positive charge in segment S4 of repeat I, yields a positive shift of about 20 mV. Stronger charge modification at the same position (K226D and K226E) produces a comparable or an even larger shift in the same direction. A larger positive shift likewise results from neutralization of the positive charges at both positions 223 and 226 (R223Q·K226Q). Reduction of positive charges in segment S4 of repeat II, for example, K859Q·K862Q, also causes a positive shift. The effects of neutralizing positive charges in repeats I and II (K226Q·K859Q·K862Q) seem to be cumulative. On the other hand, some mutations, for example, R220Q and R217Q·R220Q, evoke a shift in the opposite direction. In general, neutralizations on the C-terminal side of segment S4 (positions 223 and 226) tend to cause a positive shift, whereas neutralizations on the N-terminal side (positions 217 and 220) tend to cause a negative shift. These shifts in $V_{1/2}^m$ could arise from modifications in the effective electric field experienced by the voltage-sensing structures. A positive shift is expected from neutralization of positive charges near the intracellular surface of the membrane, whereas a negative shift is expected from neutralization of positive charges near the extracellular surface²³. Interpreting our results in terms of electrostatic effects

TABLE 1 Properties of wild-type and mutant sodium channels expressed in *Xenopus* oocytes

Mutant	ΔQ	Activation			Inactivation			I_{mean} (pA)
		$V_{1/2}^m$ (mV)	z_m (e_0)	n	$V_{1/2}^h$ (mV)	a_h (mV)	n	
Wild-type	0	-32 ± 7	2.1 ± 0.2	13	-61 ± 9	10.4 ± 1.3	9	704 (79–1,676)
R217Q	1	-34 ± 7	2.1 ± 0.3	6	-66 ± 8	9.6 ± 1.1	6	894 (200–1,573)
R220Q	1	-40 ± 10	1.5 ± 0.1	4	-67 ± 21	9.9 ± 0.8	4	461 (91–1,244)
R223Q	1	-20 ± 9	1.6 ± 0.2	5	-55 ± 14	11.8 ± 2.8	4	302 (99–644)
K226Q	1	-13 ± 2	1.8 ± 0.2	5	-67 ± 6	11.3 ± 1.0	5	1,373 (107–3,673)
K226E	2	-3 ± 6	1.2 ± 0.1	3	–71	9.9	2	470 (31–1,079)
K226D	2	-12 ± 10	1.5 ± 0.2	3	-69 ± 14	10.4 ± 2.2	3	226 (42–456)
K226R	0	-32 ± 4	2.1 ± 0.2	5	-70 ± 7	10.4 ± 1.7	4	543 (43–1,314)
S229R	–1 (0)	-25 ± 3	2.1 ± 0.2	3	-61 ± 5	10.4 ± 0.9	3	327 (120–462)
R217Q·R220Q	2	-51 ± 5	1.2 ± 0.2	5	-87 ± 5	8.6 ± 0.9	3	680 (166–1,634)
R217Q·R223Q	2	-49 ± 2	1.3 ± 0.1	4	-78 ± 2	8.6 ± 0.9	4	524 (372–665)
R217Q·K226Q	2	-14 ± 10	1.5 ± 0.1	4	-83 ± 7	9.6 ± 1.8	3	350 (100–501)
R220Q·R223Q	2	-28 ± 16	1.6 ± 0.2	4	—	—	—	153 (116–199)
R220Q·K226Q	2	-14 ± 5	1.4 ± 0.1	3	-61 ± 3	10.8 ± 2.8	3	349 (95–762)
R223Q·K226Q	2	-6 ± 6	1.4 ± 0.2	4	-70 ± 14	12.4 ± 1.2	3	209 (40–652)
K226R·S229R	–1 (0)	-25 ± 4	2.0 ± 0.1	4	-76 ± 10	9.2 ± 0.7	4	304 (92–403)
S229K·P232R	–2 (0)	-17 ± 7	1.9 ± 0.1	4	-63 ± 8	9.9 ± 1.2	4	610 (394–1,173)
R217Q·R220Q·R223Q	3	-44 ± 13	1.2 ± 0.2	3	–74	10.8	2	258 (108–385)
R217Q·R220Q·K226Q	3	-41 ± 7	1.4 ± 0.2	4	-82 ± 12	13.1 ± 3.4	3	342 (90–498)
K862Q	1 (0)	-22 ± 5	2.1 ± 0.1	5	-70 ± 7	9.6 ± 0.7	4	1,023 (456–1,903)
K859Q·K862Q	2 (0)	-5 ± 7	2.1 ± 0.2	6	-60 ± 12	13.1 ± 3.4	5	159 (20–339)
K226Q·K859Q·K862Q	3 (1)	11 ± 9	1.8 ± 0.3	5	-83 ± 8	8.9 ± 1.0	4	67 (25–170)
ΔN	0	-31 ± 3	2.0 ± 0.2	5	-68 ± 11	13.8 ± 1.5	5	130 (46–289)
cX-1	0	—	—	4	—	—	4	—
cY-1	0	-46 ± 7	2.0 ± 0.3	3	-75 ± 10	11.8 ± 2.3	3	262 (66–900)
cY-2	0	-33 ± 2	2.0 ± 0.1	3	-78 ± 5	13.1 ± 1.4	3	333 (119–529)
ΔY	0	-31 ± 3	2.2 ± 0.2	5	-71 ± 8	12.4 ± 1.2	5	103 (66–141)
cZ-1	0	-36 ± 6	2.2 ± 0.2	4	-60 ± 7	13.8 ± 1.5	3	65 (37–105)
cZ-2	0	-37 ± 4	1.8 ± 0.1	3	–61	11.3	2	131 (86–158)
ΔC	0	-40 ± 8	2.2 ± 0.2	5	-80 ± 9	8.3 ± 1.1	3	180 (70–331)

Data are given as means $\pm$ s.d. for the wild-type sodium channel and the sodium channels with point mutations in segment S4 of repeats I and/or II (upper part) and for those with a deletion, a cut or a cut/addition (lower part). All the data are taken from cell-attached macro-patch recordings. ΔQ is the reduction in positive charge caused by the mutation; the values in parentheses are the reduction in positive charge at amino-acid positions 217, 220, 223 and 226 of segment S4 of repeat I. $V_{1/2}^m$ and $V_{1/2}^h$ are the single-gate equilibrium potentials of activation and inactivation. z_m , Valence of the apparent single-gate charge for activation; a_h , the slope factor, which is inversely proportional to the maximal derivative of the inactivation curve (see Fig. 2 legend); the average values for z_m and a_h are rounded to two digits. n , Number of oocytes used, taken from at least two different series of successful injections; several patches were obtained from most oocytes. I_{mean} is the mean peak sodium current observed; the range of peak currents is given in parentheses to show the variability in the level of expression from one batch of oocytes to another. No inactivation data are available for R220Q·R223Q. cX-1 yielded currents too small to allow reliable measurements. mRNAs specific for the wild-type and mutant sodium channels were synthesized *in vitro* using *SalI*-cleaved plasmids as templates¹⁶. *Xenopus laevis* oocytes were injected¹⁷ with the wild type ($0.2 \mu\text{g } \mu\text{L}^{-1}$) or a mutant mRNA (0.2 – $0.5 \mu\text{g } \mu\text{L}^{-1}$; total concentration of an equimolar mixture for c-type mutants) and incubated¹⁷ for 4–7 days. Measurements of macroscopic currents in cell-attached membrane patches were made as described previously^{17,27}.

is qualitatively consistent with current models of the orientation of segment S4 with respect to the membrane^{5,6,24–26}.

In addition to the purely electrostatic effects mentioned above, point mutations are expected to affect the equilibrium between different channel conformations by modifying interactions between amino-acid residues. A shift in the potential dependence of activation could be explained by assuming that only one of the voltage sensors is predominantly affected, either becoming a determinant of voltage-dependent activation (positive shift) or becoming fully activated in the potential range where the activation of the other sensors occurs (negative shift). It is interesting in this context that large shifts in $V_{1/2}^m$ in both directions are usually accompanied by strong reductions in z_m for sodium channels with mutations in segment S4 of repeat I (K226E, R217Q·R220Q and R217Q·R223Q). In the case of a positive shift, the voltage sensor affected dictates the potential sensitivity since the other sensors are already at least partially activated. Because this one voltage sensor carries only a fraction of the total gating charge, the slope of the potential dependence of activation would be reduced. In the case of a negative shift, the potential-dependent activation would be dictated by fewer voltage sensors, also giving a smaller apparent z_m . However, any simplistic molecular interpretation, based on three identical independent activation gates, should be taken with reservations.

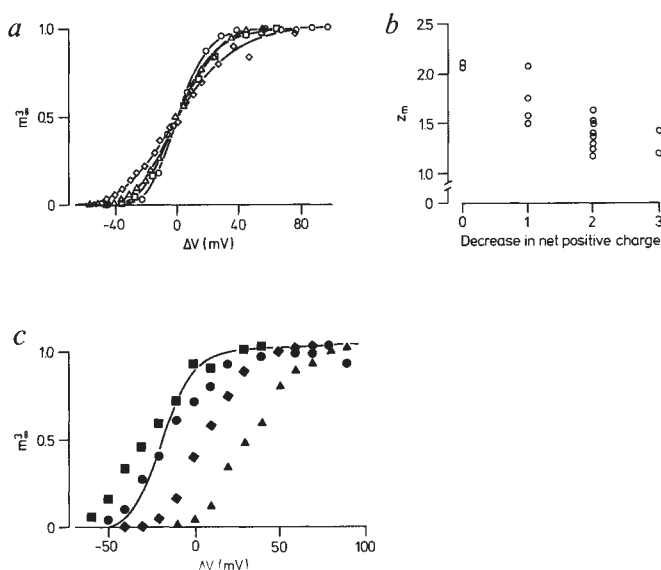


FIG. 3 *a*, Comparison of the steepness of the voltage dependence of steady-state activation (m_∞^3) for the wild-type (○) and the mutant sodium channels K226Q (□), R217Q·K226Q (Δ) and R217Q·R220Q·R223Q (◇). Single representative experiments are shown. For the purpose of making changes in slope readily visible, the activation curve of each mutant has intentionally been shifted along the voltage axis to have the same voltage of half activation as the wild-type. The continuous lines correspond to the best fits of the data points (see Fig. 2 legend). The valence of the apparent single-gate charge, z_m , is 2.1, 1.7, 1.5 and 1.1, respectively. These values represent the valence of the gating charge contributed by a single gate if a hypothetical channel comprising three identical gating subunits is assumed. *b*, Changes in z_m with decreases in the total number of net positive charges in segment S4 of repeat I at amino-acid positions 217, 220, 223 and 226. The values plotted as z_m are the average z_m values given in Table 1, but with one more digit to avoid overlapping of similar data points. Replacement of a positively charged residue by a negatively charged one is counted as a decrease of two net positive charges. *c*, Shifts of the voltage dependence of steady-state activation (m_∞^3) for mutant sodium channels. The continuous lines represent the best fit of the data for the wild-type sodium channel, assuming the following parameters for activation: a single-gate equilibrium potential ($m_\infty = 0.5$, $V_{1/2}^m$) of -32 mV and a z_m value of 2.1. Single representative experiments for the mutant sodium channels are shown and their $V_{1/2}^m$ (in mV) and z_m values are as follows: R217Q·R220Q (■), -53 and 1.2; R220Q (●), -38 and 1.4; K226Q (◆), -13 and 1.7; K226Q·K859Q·K862Q (▲), $+10$ and 1.6.

The potential dependence of the time constants of activation and inactivation for the wild-type and the mutant K226Q is shown in Fig. 2*b*. It is seen that for this mutant the voltage dependence of τ_m and τ_h shows a shift similar to that of $V_{1/2}^m$. The steady-state properties of inactivation are not systematically affected by the mutations in segment S4 of repeat I (Table 1 and Fig. 2*d*).

Expression of deletion mutants

The presence of four internal repeats suggests that the sodium channel evolved by duplications of an ancestral gene⁵. To examine whether individual repeats or their combinations can form functional sodium channels, we prepared mRNAs encoding single repeats or several contiguous repeats by transcription *in vitro* of the corresponding cDNAs (Fig. 1*c*). Δ -Type mutants were produced by injection into *Xenopus* oocytes of an mRNA encoding the sodium channel protein with a deletion. c-Type mutants were generated by injection of an equimolar mixture of two mRNAs encoding adjacent fragments of the sodium channel protein separated by a cut, either with an addition of four to eight residues at each end of the cut (cut/addition) or with substitution of the initiating methionine for the N-terminal residue of the C-terminal fragment (cut). mRNAs encoding only repeat I (derived from the plasmid pADI-1 or pADI-2; see Fig. 1 legend), repeat IV (pADIV-1 or pADIV-2), repeats I/II (pADI/II-1 or pADI/II-2), repeats III/IV (pADIII/IV-1 or pADIII/IV-2), repeats I/II/III (pADI/II/III-1 or pADI/II/III-2) or repeats II/III/IV (pADII/III/IV-1 or pADII/III/IV-2) did not give rise to sodium currents that could be measured using the two-electrode voltage clamp. Combinations of two mRNAs encoding repeat I and repeats II/III/IV (cX-1, cX-2 and cX-3 each having a cut/addition at different positions between repeats I and II; see Fig. 1*c* and legend) produced marginal sodium currents. By contrast, significant expression of sodium currents was observed for the mutants with a cut/addition (cY-1 and cY-2) or a deletion (Δ Y) between repeats II and III, the mutants with a cut/addition (cZ-1) or a cut (cZ-2) between repeats III and IV and the mutants with a

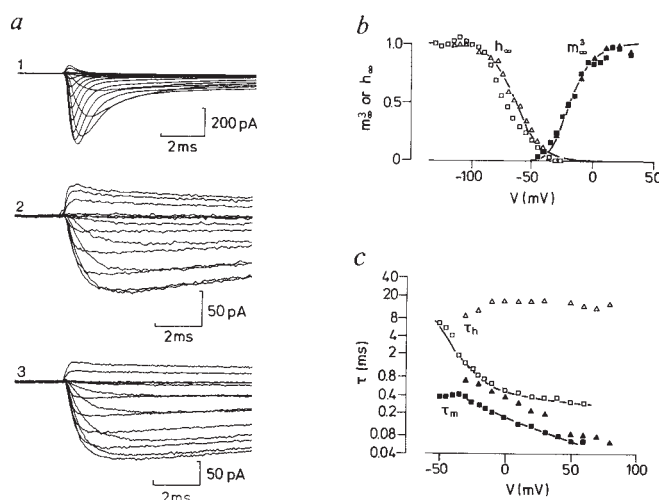


FIG. 4 *a*, Current responses to depolarizations in oocytes injected with the mutant sodium channel cY-2 (1) (0.33 $\mu\text{g } \mu\text{l}^{-1}$), cZ-1 (2) (0.33 $\mu\text{g } \mu\text{l}^{-1}$) or cZ-2 (3) (0.5 $\mu\text{g } \mu\text{l}^{-1}$) mRNA. Responses were evoked by depolarizations ranging -60 – $+70$ mV ($+90$ mV for 1) in 10 mV steps from a holding potential of -120 mV. Averages of 16 individual traces. Temperature: 15°C . *b*, Voltage dependence of steady-state activation (m_∞^3 , filled symbols) and inactivation (h_∞ , open symbols) for cY-2 (■, □) and for cZ-1 (▲, △). The smooth lines represent the best fit of the data for the wild-type in Fig. 2*d*. *c*, Voltage dependence of the time constants of activation (τ_m , filled symbols) and inactivation (τ_h , open symbols) for the same mutant channels as in *a* and *b*. The continuous lines correspond to the wild-type data in Fig. 2*b*.

deletion in the N-terminal (ΔN) or the C-terminal (ΔC) region. These results suggest that all four repeats are required for the formation of functional sodium channels.

Effects on inactivation

In Fig. 4a, macroscopic currents recorded from oocytes implanted with the mutant cY-2 (1), cZ-1 (2) or cZ-2 (3) are shown. Apart from their magnitude, the currents produced by the mutant cY-2, which has a cut/addition between repeats II and III, are similar to the wild-type (Fig. 2a, 1). By contrast, the currents evoked by the mutants cZ-1 and cZ-2, which have a cut/addition or a cut, respectively, between repeats III and IV, are characterized by a dramatic decrease in the rate of inactivation. A quantitative comparison of the steady-state and kinetic properties of cY-2 and cZ-1 with those of the wild-type channel is shown in Fig. 4b, c. For both mutants, the steady-state properties of activation and inactivation are similar to those of the wild-type. However, the τ_m of the mutant cZ-1 increases by a factor of about 1.8 and the τ_h of the mutant cZ-1 becomes nearly voltage-independent, being about 30-fold greater at strong depolarizations than that of the wild-type.

To characterize the properties of the mutant cZ-1 in greater detail, we measured the currents flowing through single channels, using smaller-diameter patch pipettes^{17,27}. By contrast with the wild-type channel which exhibits short openings clustered at the beginning of the depolarization¹⁷, openings sometimes lasted for periods as long as the voltage step (80 ms) (Fig. 5a). The open-time histogram for elementary currents flowing through the mutant cZ-1 channel is shown in Fig. 5b. The data are well fitted by a single exponential with a mean open time of 5.8 ms. Similar values were obtained over the potential range

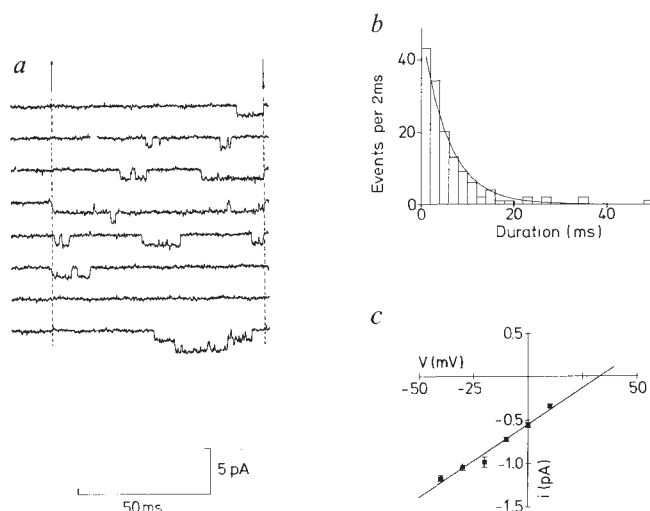


FIG. 5 a, Single-channel currents recorded from a patch on an oocyte implanted with the mutant sodium channel cZ-1. Responses to successive 80-ms depolarizations to -20 mV from a holding potential of -100 mV are shown. The dashed lines indicate the beginning and end of the pulse. The closed channel current level is the same as that before the start of the pulse. The original data were sampled at intervals of $50 \mu s$ and filtered at 4 kHz (-3 dB). Leakage subtraction was performed as follows. A fourth-degree polynomial was fitted to the current after the decline of the capacitive transients, for an average of eight hyperpolarizing pulses to -140 mV. The first few microseconds of the capacitive transients were left unchanged. This composite trace was then scaled and subtracted from each original trace. This method of leak subtraction introduces significantly less noise than conventional methods. Subsequently, the data were digitally filtered with a gaussian filter at 2 kHz (ref. 32). b, Distribution of open times from 140 single-channel events recorded at -20 mV. Openings shorter than $500 \mu s$ were disregarded. The distribution is fitted by a single exponential with a decay time constant of 5.8 ms, using a maximum likelihood method. c, Single-channel current-voltage relation. The straight line corresponds to a slope conductance of 17.3 pS.

-60 to $+10$ mV. By contrast, the mean open time of the wild-type channel measured at -32 mV is more than one order of magnitude smaller¹⁷. Single-channel current amplitudes measured for the cZ-1 channel in the potential range -40 to $+10$ mV are plotted in Fig. 5c. The slope conductance of 15.7 pS (mean of two experiments) obtained for the cZ-1 channel is reasonably close to the value of 19 pS estimated for the wild-type channel¹⁷.

The drastic slowing of the decay phase of macroscopic sodium currents and the increase in the mean open time of single-channel currents observed for the mutant cZ-1 are similar to the effects reported for native sodium channels treated with intracellularly applied endopeptidases^{22,28,29}. We tested the action of trypsin on the sodium channel expressed in oocytes injected with the wild-type mRNA. In inside-out patches, a nearly complete removal of sodium current inactivation was observed after treatment with trypsin ($50 \mu g\ ml^{-1}$; for 10 min at $16^\circ C$) (data not shown). An increase in peak sodium current was also seen after removal of inactivation, as described for the squid axon²².

It has been proposed that the four internal repeats of the sodium channel, containing all putative transmembrane segments, are linked by three relatively large hydrophilic regions (53–326 amino acids in length in rat sodium channel II; see ref. 6). All these regions, together with the hydrophilic N- and C-terminal regions, are assigned to the cytoplasmic side of the membrane. Although cleaving the link between repeats III and IV affects mainly the rate of inactivation, the integrity of the link between repeats II and III seems to be of less functional importance. Neither cleaving the link between repeats II and III (cY-1 and cY-2), nor making a relatively large deletion in this region (ΔY) causes appreciable effects (Table 1). This is also the case for a deletion in the N-terminal (ΔN) or the C-terminal (ΔC) region (Table 1). These results indicate that the region between repeats III and IV is important for the inactivation of the sodium channel and that this region is likely to contain the sites at which intracellular proteolytic digestion exerts its effects. Because the mutants with a cut/addition in the region between repeats I and II fail to exhibit sodium currents large enough to allow detailed analysis, the possibility that this region is also involved in inactivation cannot be excluded. Our results are consistent with the proposed intracellular location of the region between repeats III and IV. This region contains a cluster of conserved positively charged residues (mainly lysine) and has been proposed to be involved in the inactivation of the sodium channel⁶.

Discussion

Functional analysis of mutated sodium channels expressed in *Xenopus* oocytes allows us to gain several types of information about the structure-function relationship of this voltage-gated ionic channel. Our results show that reducing the net positive charge in segment S4 of repeat I causes a decrease in the apparent gating charge, as manifested by a reduction in the steepness of the potential dependence of activation. This finding provides experimental evidence that the positive charges in segment S4 of repeat I constitutes at least part of the gating charge involved in the voltage-dependent activation of the sodium channel. The unique structural features of segment S4 are strikingly well conserved in the calcium channel^{9,10} and the potassium channel^{11–15}, which show amino-acid sequence homology with the sodium channel. This implies that the involvement of the clustered positive charges in segment S4 represents a common mechanism responsible for the activation of voltage-gated ionic channels.

Our results also show that cleavage of the linkage between repeats III and IV of the sodium channel causes a strong reduction in the rate of inactivation. This finding, together with the similar effect observed for the wild-type sodium channel treated with intracellularly applied endopeptidases, supports the view that this region, located on the cytoplasmic side of the

membrane, is involved in the inactivation of the sodium channel.

Another finding of the present investigation is that the homologous internal repeats of the sodium channel can function as structural units to form functional channels, even when a linkage between the repeats is cleaved. However, unlike the potassium channel which has a basic structure corresponding to a single repeat of the sodium channel^{11,13-15} and is assumed to form a homo-oligomeric complex¹², all four repeated units

are required for the formation of functional sodium channels. This suggests that, during the evolution of the sodium channel by gene duplications, each of the four repeated units may have acquired a differentiated function. □

Note added in proof: Since the submission of the first version of this manuscript, it has been reported that antibody directed against a peptide sequence in the region between repeats III and IV slows inactivation of the sodium channels³³.

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LETTERS TO NATURE

Intense 6.7-keV iron line emission from the Galactic Centre

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THE Galactic Centre, although obscured by dust at optical wavelengths, is luminous in higher-energy radiation. Gamma-ray observations suggest that a large number of supernovae or novae, or a single supermassive object, has exploded in the Galactic Centre¹. Such an explosion will heat up the interstellar medium, probably creating an optically thin hot plasma. Here we report the discovery of intense iron-line emission at 6.7 keV from the Galactic Centre, detected by the Ginga satellite, with characteristics consistent with its production by a shock-heated plasma. The line intensity peaks at the Galactic Centre with a surface brightness of $\sim 1.5 \times 10^{-7} \text{ erg s}^{-1} \text{ sr}^{-1} \text{ cm}^{-2}$ and extends over an angular size of 1.8° . The total luminosity of the iron line at the centre region is $\sim 2.1 \times 10^{36} \text{ erg s}^{-1}$ assuming that the distance to the centre is 10 kpc. The line intensity at the centre is about ten times larger than that from the region of the galactic ridge. The line feature observed is consistent with thermal emission from a thin hot plasma at a temperature of $\sim 10 \text{ keV}$. The abundance of iron is estimated to be comparable to or larger than the cosmic abundance. This thin hot plasma is attributable to a shock-heated interstellar medium by an energetic explosion.

In the X-ray continuum energy band, several point sources and unresolved diffuse emission have been discovered in the Galactic Centre region. (See, for example, refs 2-4 and references therein). However, the continuum X-ray intensity in the region of the Galactic Centre is not particularly strong compared with that in the other regions of the Galaxy, in contrast to radio, infrared and γ -ray bands. An optically thin hot plasma can

produce characteristic X-rays from highly ionized atoms. Thus, observations of characteristic X-rays may provide a new clue to activity near the Galactic Centre, and we therefore carried out X-ray mapping of iron K-line emission along the galactic plane.

The galactic plane was scanned with the large-area proportional counters (LAC)⁵ on board the X-ray astronomy satellite Ginga⁶ on several occasions: August and October in 1987 and March in 1988. The total effective area of the LAC is $4,000 \text{ cm}^2$, with a field of view (or beam size, full width at half maximum) of 1° along and 2° perpendicular to the scan path. Apart from point sources, we see no time variability of X-ray flux during these three scans, and these scan data, within 0.5° of the plane, were superposed to construct the X-ray profile along the galactic longitude. In Fig. 1a, we show the X-ray intensity profile on the galactic plane in the continuum energy band 2-18 keV. We see many prominent peaks corresponding to known binary X-ray sources (but no prominent peaks are seen at the Galactic Centre).

We then sliced the scan data and constructed the X-ray spectrum for each position on the galactic plane (to 0.4° resolution). Figure 2 shows the spectrum near the Galactic Centre. An intense emission-line feature at 6.7 keV is observed, as was already noted by Koyama⁷. In order to evaluate the line energy and intensity from the galactic plane, we fitted a model assuming a free-free spectrum for the continuum and a narrow emission line. Most of the data, except for the data near bright X-ray binary sources, can be fitted with this model. Using the acceptable results, we plotted the best-fit intensity and energy of the emission line against galactic longitude, as shown Fig. 1b, c. A remarkable feature discovered here is a strong peak in line intensity near the Galactic Centre. Here, the line intensity is stronger than those from the bright point sources (the average intensity of the iron line at the peaks of the six brightest sources is $\sim 70\%$ of that of the Galactic Centre), although they are not shown because of large statistical errors resulting from strong continuum intensities. The broad hump near galactic longitude $l = 30^\circ$ corresponds to the '5 kpc ring' where the CO-line and infrared and γ -ray intensities show local maxima⁸. The peak at $l = 25^\circ$ and complex structure near $l = 350^\circ$ are due to point sources. The energy of the emission line is consistent with 6.7 keV, suggesting that it corresponds to the K-line of helium-

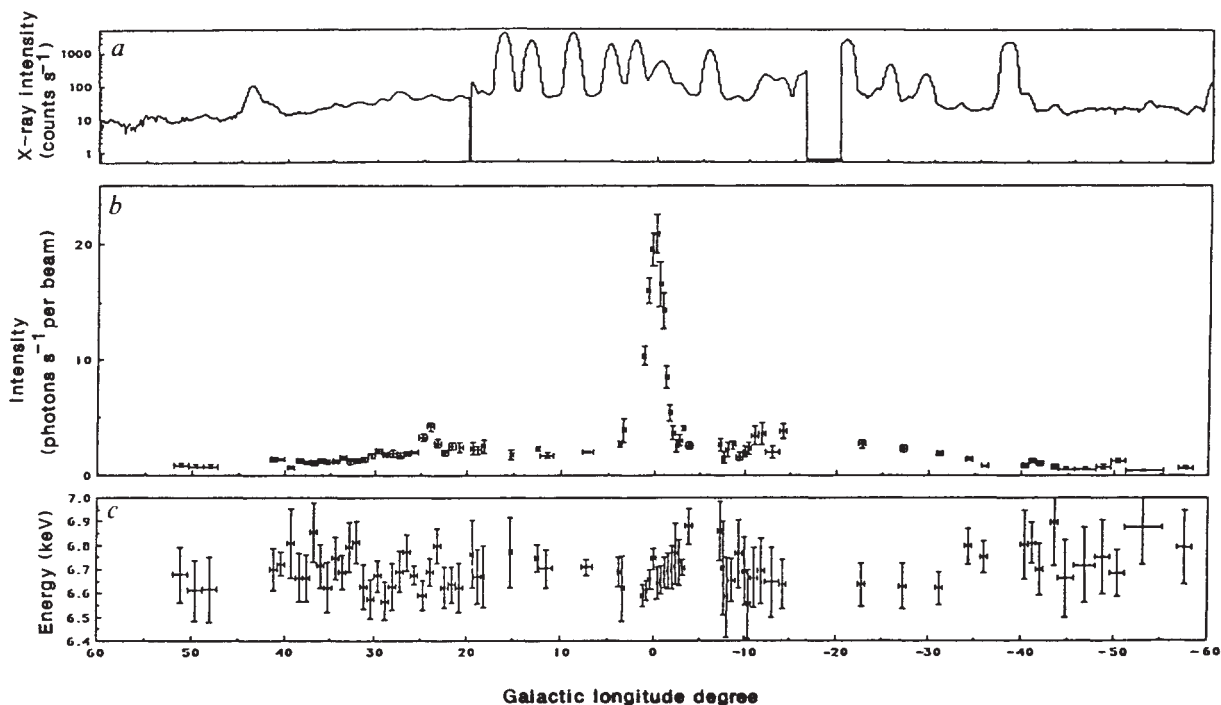


FIG. 1 The scan profile of the X-ray emission in the 2–18-keV energy range (a) and that of the iron-line emission (b) on the galactic plane. The line energy is plotted in c.

like iron. Helium-like iron is the most abundant ionization state in a plasma of which the temperature ranges from several to 10 keV. In fact, the plasma temperature determined independently from the continuum spectra in point-source-free regions is ~ 10 keV. Thus the observed iron-line feature strongly supports the idea that the origin of the X-ray emission is due to an optically thin hot plasma at a temperature of ~ 10 keV. A thin hot plasma with the same temperature range has been reported from the galactic ridge ($30^\circ < l < 60^\circ$; ref. 9) and is clearly confirmed by these observations. The newly discovered peak of the iron-line flux at the Galactic Centre is about ten times brighter than that from the galactic ridge.

Figure 3 comprises enlarged sections of Fig. 1 near the Galactic Centre. The beam size (collimator response function) at the Galactic Centre is also given by a solid line in Fig. 3b. The peak of the continuum emission is offset by $\sim 0.5^\circ$ from the Galactic Centre (corresponding to the position of the low-mass binary source A1742–29) and the intensity distribution is asymmetric, possibly as a result of several faint sources and/or diffuse emission. By contrast, the intensity peak of the iron line is

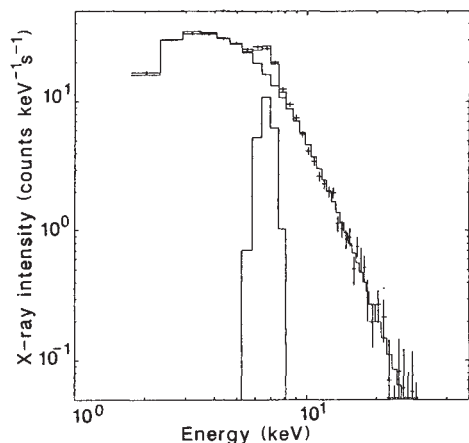


FIG. 2 The X-ray spectrum near the Galactic Centre together with the best-fit model of free-free plus line emissions.

located at the Galactic Centre and the intensity distribution is almost symmetric. As the width of the iron-line scan profile is larger than the beam size, this central iron-line emission is not due to a single point source at the Galactic Centre but to a superposition of many sources and/or diffuse emission symmetrically distributed with respect to the Galactic Centre. The angular size of the extended line emission is $1.8^\circ \pm 0.1^\circ$ corresponding to 300 pc (full width at half maximum) and the total luminosity of the iron line is estimated to be $(2.3 \pm 0.1) \times$

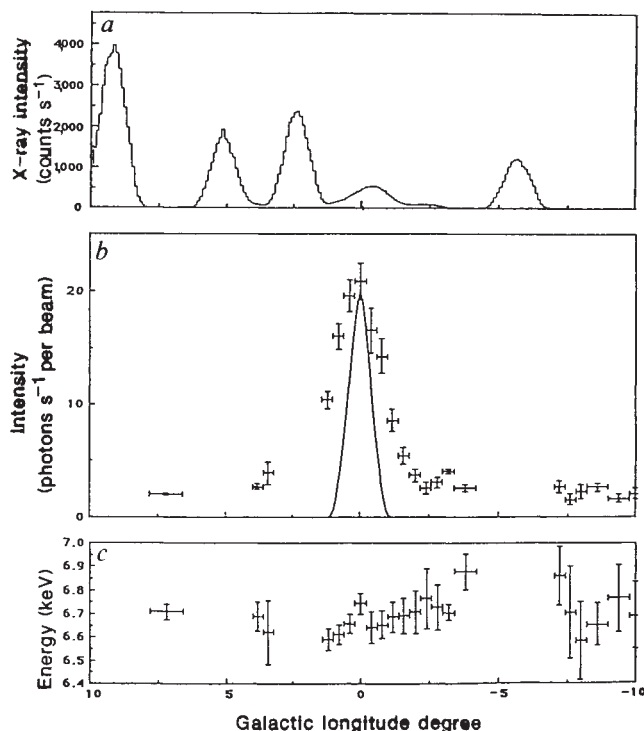


FIG. 3 Same as Fig. 1, but near the Galactic Centre. The collimator response at the Galactic Centre is shown as a solid line in b.

$10^{36} \text{ erg s}^{-1}$ assuming a spherical gaussian distribution around the Galactic Centre at a distance of 10 kpc. The surface brightness of the iron line at the peak is $(1.5 \pm 0.2) \times 10^{-7} \text{ erg s}^{-1} \text{ sr}^{-1} \text{ cm}^{-2}$.

To estimate the luminosity of the extended emission in the continuum X-ray band (2–10 keV) of the line-emitting plasma, we carried out a model convolution fit to the scan profile (2–10 keV band) of $\pm 1.5^\circ$ region near the centre, including the extended emission and the three brightest point sources, A1742–294, 1E1740.7–2942 and 1E1743.1–2843 (refs 3, 4). For the extended continuum emission we assumed the same profile as that of the iron-line emission. There are, therefore, four free parameters in the model, namely the intensities of the three brightest point sources and the extended emission. From the best-fit result, we find the total flux of the extended emission to be $\sim 4 \times 10^{37} \text{ erg s}^{-1}$ (2–10 keV) with a temperature of 10 keV and a distance of 10 kpc. The surface brightness at the peak is $\sim 3 \times 10^{-6} \text{ erg s}^{-1} \text{ sr}^{-1} \text{ cm}^{-2}$ (2–10 keV), from which we estimate the equivalent width of the iron line from the extended emission to be $600 \pm 70 \text{ eV}$. This is consistent with the value expected from a 10 keV plasma of cosmic abundances¹⁰ in the collisional ionization equilibrium. As we neglected the contribution of other unresolved point sources in the model fitting, our estimated intensity of the extended continuum emission may have significant uncertainty. In fact, the peak flux of the extended continuum emission is roughly consistent with those of refs 3 and 4 but the total flux is larger. The inconsistency arises because the spatial extension of our result is much larger than that in refs 3 and 4. We re-estimate the iron abundance using the continuum emission from refs 3 and 4 and the iron intensity observed here. We find that the iron abundance at the Galactic Centre is about 1–2 times the cosmic abundance.

As mentioned above, the total intensity of the continuum X-ray and iron abundance from the extended emission are subject to significant uncertainty. We conclude, however, that the extended emission is due to an optically thin hot plasma produced by a collisional process. This suggests that the extended emission arises from a hot plasma shock-heated by an energetic explosion. $\square$

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Creation of the Uranus rings and dust bands

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VOYAGER observations of the extended hydrogen exosphere of Uranus and of the ϵ Ring and its shepherds set an upper limit to the age of the ϵ Ring of 6×10^8 years. Unless we are seeing Uranus at a special time in its history, this requires a continuing process to create the ring material. We propose that the moons, rings and dust now visible in the Uranus system are created by the diminution of larger objects by meteoroid impacts. A Monte Carlo calculation shows that the largest surviving fragments of the original precursor

satellites are in the size range of the new satellites observed by Voyager near the rings. The current bombardment rate is sufficient to create all the observed rings and dust bands. The observed and inferred components of the Uranus ring system fit a power-law size distribution with index of ~ 3 . We predict that the material in Neptune's ring system has a similar size distribution.

We first derive a stringent upper limit to the age of the ϵ Ring from the drag of the hydrogen exosphere on the rings¹. The exospheric drag provides a lower limit to the torques transferred to the satellites shepherding the ϵ Ring. Because the ϵ Ring outer edge is located at the 14:13 inner eccentric resonance of the satellite Ophelia, the viscous torque T_v (which causes the ring to spread from the effects of mutual ring-particle collisions) must exceed the torque from the exosphere, T_D . Otherwise the edge would be blown inwards as the ring particles move through the exosphere². The excess momentum, $T_v - T_D$, is carried off by the resonance coupling to Ophelia. This momentum transfer causes Ophelia to evolve outwards, away from the ring. The minimum viscous torque is thus $T_v = T_D$.

The ϵ Ring inner edge is located at the 24:25 outer eccentric resonance of the satellite Cordelia³. This resonance transfers the momentum of the inwardly diffusing ring particles to Cordelia. At the inner edge, this momentum transfer, $T_v + T_D \geq 2T_D$, causes Cordelia's semi-major axis to decrease. As the mass of Cordelia is known³, we can estimate how long it would take Cordelia to evolve to its current separation, assuming the minimum viscous torque and zero initial separation. The maximum duration of this shepherding is thus $t_{\text{max}} = \Delta L / (2T_D)$, where ΔL is the total change in angular momentum and $T_D = 9 \times 10^{16} \text{ erg}$ (ref. 2), and hence $t_{\text{max}} = 6 \times 10^8 \text{ yr}$. Cordelia could not have transferred this momentum to a larger inner moon as none exists. Further, it could not have transferred it to one of the larger outer moons through a resonance that may have existed in the past as capture into a resonance libration with an outer moon is not possible⁴ given the tidal expansion of the orbit of the larger outer satellite and the shrinking of Cordelia's orbit. This age, t_{max} , is a strong upper limit because we have used minimum values for the torque, T_v . Similar lifetimes have been inferred for the small moons discovered by Voyager⁵. The lifetime of the dust seen in the Voyager forward scattering image⁵ is even shorter, $t \leq 1,000 \text{ yr}$ (ref. 1). Thus, all the components of the Uranus ring system appear significantly younger than the Solar System. Our proposed solution to these short lifetimes is that the dust, rings and small moons are created continually by disruption of larger objects.

Consider the dust bands first. Because they are not all coincident with the main uranian rings, these rings cannot be their only source. We interpret the dust bands as the visible component of moonlet belts whose opacities are otherwise too small to be visible to the Voyager instruments. Similarly, dust bands are created by collisions and erosion in each of the main rings. Each moonlet belt could be composed of objects $\sim 100 \text{ m}$ in radius with a belt width of tens of kilometres and an optical depth of 10^{-3} or less, similar to that proposed for Saturn's F Ring⁶. Dust particles are ejected from the belt objects by meteoroid impacts and collisions between belt objects, and they travel on ballistic trajectories until sticking on the surface of another moonlet or escaping the belt by exospheric drag.

The large moonlet-belt bodies and the ring precursor satellites also serve as targets for meteoroids large enough to release enough dust from a single impact to be observed as a dust band. Such a dust band would radially evolve and dissipate on a short timescale ($\leq 100 \text{ yr}$) because of gas drag, and it would tend to be broader, with more diffuse edges, than the dust component of a main ring or moonlet belt. In addition, continuous removal of meteoroid ejecta from rings, moons and moonlet belts by gas drag leads to a continuous low-optical-depth sheet of dust in the main ring system, with the highest optical depth near the ϵ Ring, where gas drag is slower. In our model, these processes and variations in the widths, optical depths and size distributions

of the moonlet belts account for the variety of forms of dust bands seen by Voyager.

We calculate the optical depth, τ , of meteoroid ejecta in a moonlet belt:

$$\tau = \frac{T_{\text{ff}}}{A'} \tau_{\text{MB}} \iint \pi a^2 n(m, a) A f(m) da dm \quad (1)$$

where $T_{\text{ff}} = 2\pi/(\Omega\tau_{\text{MB}})$ is the time of flight of the ejecta between release from one object and capture on another, and Ω is the orbital frequency; τ_{MB} is the optical depth of the objects in the moonlet belt, $n(m, a) da$ is the number of particles in the size interval $[a, a+da]$ released from an impact by a meteoroid of mass m , A' is the area over which meteoroid ejecta are spread (larger than the area of the belt itself, A , because of the distribution of ejecta orbits⁷), and $f(m) dm$ is the number flux of impactors in the mass interval $[m, m+dm]$. For $f(m)$ we use the interplanetary micrometeoroid flux at 1 AU (ref. 8) scaled to 19 AU as suggested by Grün *et al.*⁸ and extended by a power law such that the largest meteoroids would produce the number and slope of small craters on the uranian satellites^{5,9}. For $n(m, a)$ we assume a power law with index $q = 3.5$, and we assume the ejecta mass is proportional to the kinetic energy of the impact¹⁰. We obtain $\tau \approx 5 \times 10^{-6}$ for all $\tau_{\text{MB}} > 10^{-5}$. For smaller τ_{MB} the ejecta are not recaptured but are removed from the belt by gas drag, and τ decreases linearly with τ_{MB} .

Additional dust is released by collisions between moonlet-belt objects⁶. The mass influx per second due to collisions is balanced by the sweep-up of ejecta by the same moonlet-belt objects. For $\tau_{\text{MB}} = 10^{-4}$, a power-law size distribution of moonlet-belt bodies with index 3 and largest body 500 m, and relative velocities of 20 cm s^{-1} (approximately the escape velocity of a 200-m body), we calculate $\tau \approx 10^{-5}$ when we include meteoroid ejecta and collisions, consistent with the range of observed dust optical depths^{5,11}. Similar values of τ are obtained for the main rings. To match Voyager observations we require one moonlet belt for each observed dust band that is not coincident with a classical ring, and $\tau_{\text{MB}} < 10^{-3}$ (refs 5, 12). Thus we predict that there are ~ 50 belts with $10^{-5} \leq \tau_{\text{MB}} \leq 10^{-3}$.

We suggest from our model that the moonlet-belt objects and the main rings of Uranus have similarly been created by the break-up of larger objects. For the rings, we propose that the impact event was more disruptive and gave rise to a steeper

distribution of fragments in particle size (creating many smaller particles and explaining the enhancements seen in Fig. 1). For a continuing source of the nine main rings (with a ring lifetime of $\sim 10^8 \text{ yr}$) we require a current population of ~ 35 10-km-radius objects, each with a disruption lifetime of $\sim 350 \times 10^6 \text{ yr}$ (calculated below). These 10-km objects are produced in turn by the disruption of larger objects.

Voyager observations of craters establish the disruption rate for the larger bodies in the Uranus system. Crater counts on the uranian satellites show that the number of craters per unit area that are larger than size d , $N_{\text{cum}}(d)$, goes as d^{-3} (refs 5, 9), and similar results hold for Saturn¹³. The minimum-sized impactor for catastrophic disruption is that which creates a crater that is some constant fraction (about one half) of the satellite radius (W. B. McKinnon, personal communication). We can then estimate the lifetime T_R of a satellite of radius R against catastrophic disruption by $T_R \propto [4\pi R^2 N_{\text{cum}}(R/2)]^{-1}$, or $T_R = T_0(R/R_0)$ where T_0 is the impact lifetime of an object with radius R_0 . The smaller fragments from a catastrophic disruption are therefore destroyed more rapidly than the larger fragments. In the following analysis we shall ignore these smaller, more rapidly destroyed fragments and concentrate on the largest fragment. Because some fraction of the smaller fragments will survive¹⁴, the largest fragment surviving after a time t provides a lower limit to the amount of material remaining after t .

With $T_R \propto R$ and $T_0(R_0 = 100 \text{ km}) \approx 3.5 \times 10^9 \text{ yr}$ (ref. 5), we can estimate the largest particle $R(t)$ remaining from an original satellite of radius R_0 . After a number of disruptions, n , (each of which leaves the largest fragment roughly one half the radius of the previous object), the largest fragment radius is $R_n \approx R_0 2^{-n}$. Assuming Poisson statistics for the waiting times gives for t_n , the time to the n th disruption,

$$t_n = 2T_0 \sum_{i=1}^n 2^{-i} = 2T_0(1 - 2^{-n}). \quad (2)$$

Inserting this in the expression for R_n gives

$$R(t) = R_0 \left(1 - \frac{t}{2T_0}\right) \quad (3)$$

To check these calculations we have carried out a Monte Carlo simulation of the size evolution of an original satellite subject to consecutive disruptions. These simulations and calculations show that if originally some small number of 100-km objects were spread across the region outside the uranian rings, the impacting flux inferred from Voyager images⁵ would leave the largest fragments with a size of ~ 30 – 50 km (equation (3)). The satellite Puck were spread throughout the region now containing consider this a confirmation, but it shows no contradiction between the model disruption rate (calculated from the crater record⁵) and the observed moons near the rings. We therefore propose that perhaps 12 original moons about the size of the satellite Puck were spread throughout the region now containing the dust, rings and small moons. The gradual diminution and break-up of these objects provides the source for all the material now visible.

This simple model does not consider the orbital evolution of the larger fragments. We presume that the disruptive events spread material over a range of azimuth and radius and that these fragments are subject to torques from gravitational resonances. We ignore re-accretion of these objects.

If, as we propose, the dust-ring-moon system is a collisionally evolved system, we expect a broad size distribution similar to that for the asteroids or collisionally created fragments in laboratory experiments. These distributions are well described by a power law with index q such that $3 \leq q \leq 4$ (ref. 15). The slope of the size distribution is made shallower by gas drag preferentially removing smaller particles¹⁶, but this affects only the smallest particle sizes. In Fig. 1 we have plotted the mass and surface area of the observed segments of this distribution along

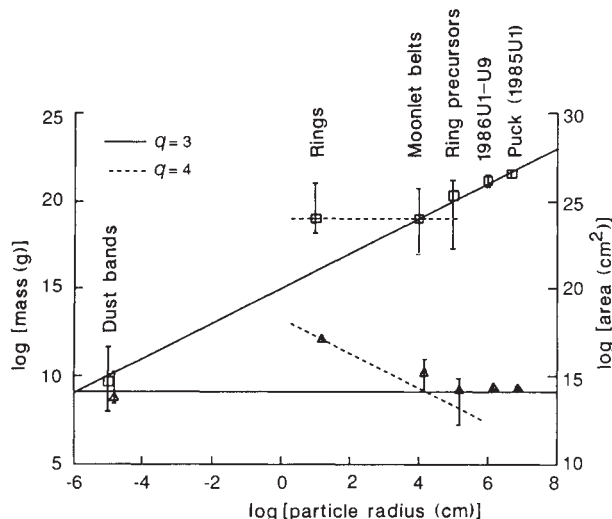


FIG. 1 Integrated surface area and mass of segments of the Uranus dust-ring-moon system. Squares: mass; triangles: surface area; lines: predictions for power-law distributions $n(a) da = Ca^{-q} da$. Error bars for the inferred unseen segments were estimated from model and observational constraints. Surface-area points have been horizontally displaced from the mass points for clarity.

with those segments inferred in this work (the 100-m moonlet-belt objects and the 10-km ring precursors). We see that the entire distribution is roughly consistent with a power law of index $q \approx 3$, and with enhancements around the main rings with $q \approx 4$. The visibility of the rings is due to their enhanced area (and optical depth), the moons to their size compared with the Voyager camera picture element, and the dust bands to their strong forward scattering of light. The rest of the distribution is unobserved.

If Neptune has a similarly evolved ring system of which the ring arcs are the only currently observed segment, we expect that Voyager will discover a retinue of small moons and dust bands. If the size distribution is also characterized by $q = 3$, then the surface areas in all segments will be roughly equal. Extrapolating from the observed arcs and the shepherds necessary to hold these arcs in place (B. Sicardy, personal communication) we predict dust bands having a total surface area of 10^{12} – 10^{14} cm². □

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Existence of moonlets in Saturn's rings inferred from the optical depth profile

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It has been suggested^{1,2} that small satellites of diameter less than 50 km (moonlets) could exist within planetary rings, and could cause 'grooves' of low density in the adjacent ring material. Here we re-examine this hypothesis by comparing the numerical simulations of the gravitational influence of a moonlet on the radial density profile^{3–5} with the occultation data collected by the Voyager ultraviolet spectrometer (UVS). We inspected by eye the UVS optical-depth profile of Saturn's rings to identify features similar to those predicted theoretically, and those examples found were analysed statistically to exclude the possibility of a chance origin. Six significant features in the radial optical-depth profile have been found, which could be caused by moonlets ranging in size between 7 km and 30 km, located mainly in the middle of the B-ring.

Despite a careful search of Saturn's rings by Voyager 2, moonlets have not been detected. It is probable that, if they exist, they are too small to have been observed directly by Voyager amidst the bright ring material⁶. Furthermore, with the exception of the density wake caused by the Encke-gap moonlet^{7,8}, the existence of such moonlets has not yet been confirmed by indirect observation of moonlet-induced density features, mainly because of insufficient knowledge about the gravitational

influence of the moonlets on the ring structure. In an attempt to address this problem we have investigated numerically the gravitational effect of a moonlet on the rings' radial density profile^{3–5}.

We have carried out numerical test-particle experiments that simulate the motion of particles in the ring plane near the orbit of a moonlet. Collisional interactions and self-gravitation of the ring are neglected, so that the dynamics of the ring particles is governed only by the gravitational fields of the planet and of the moonlet, and can be described by the plane-restricted three-body problem^{3,4}. Simulations with different masses M_m of the moonlet are not necessary, because the basic dynamic equations are nearly independent of M_m if they are scaled in Hill-sphere units³, the radius h of the Hill sphere being

$$h \approx a_m \left(\frac{M_m}{3M_p} \right)^{1/3} = a_m h_* \quad (1)$$

where a_m is the semimajor axis of the moonlet orbit and M_p the mass of Saturn. For spherical icy moonlets with a radius R_m and a mean density in the range 0.7–1.0 g cm^{−3}, the cubic root in equation (1) takes a typical value of $h_* = 10^{-5} R_m / [\text{km}]$.

Changes in the semi-major axes and eccentricities of the test particles caused by a single encounter with the moonlet were used to calculate the modification of an initially uniform particle number density^{3,4}. Many repetitions of this scattering process⁵ yield a nearly stable radial particle-density profile (Fig. 1a) after about one synodic period $t_s \approx P/h_*$, where P is the orbital period of the moonlet. This scattering structure is typical of particle ensembles having mean eccentricities $\langle e \rangle < h_*/2$. This condition is satisfied for moonlet radii $R_m > 0.5$ km.

We now consider the errors that may arise from the neglect of collisional effects and self-gravitation of the ring. The latter effect is of minor importance because the critical length⁹ L_c of free gravitational instabilities is much smaller (≈ 10 m) than the smallest density features observed by the Voyager probes. Collisional effects such as viscous spreading are, however, very important in planetary-ring dynamics¹⁰. Estimates of the influence of collisions on the stability of density gradients³ have yielded nearly stable density features for times $t > t_s$ in regions far from resonance locations of outer satellites. There, the velocity dispersion c of random particle motion, which strongly influences the viscosity ($\nu \propto c^2$), becomes^{10,11} $c < 10^{-3} \text{ m s}^{-1}$. Thus, in these regions our assumptions seem to be justified. Near resonances, which are concentrated mainly in the outer A-ring, c is a few orders of magnitude larger and density gradients will be smeared out in a time $t(c) < t_s$.

The characteristic features of the scattering structure (Fig. 1a) obtained from our computer experiments are: (1) an inner 'ringlet' of enhanced density within $|b| < 1.2$ (here b is the radial

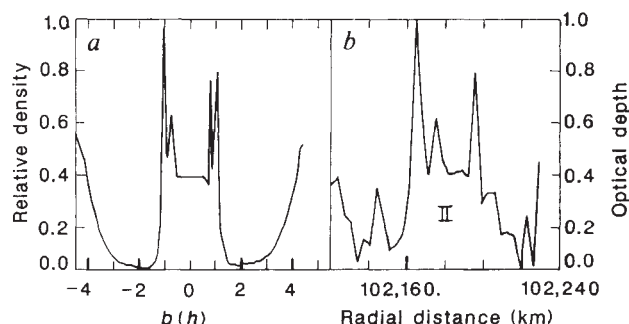


FIG. 1 a, Surface particle density calculated by computer experiment (in relative units) as a function of the radial distance $b(h)$ from the moonlet orbit. The particle density σ is normalized to its minimum and maximum according to $(\sigma - \sigma_{\min})/(\sigma_{\max} - \sigma_{\min})$, with $\sigma_{\min} = 0.1$ and $\sigma_{\max} = 2.67$. b, The optical depth τ , normalized according to $(\tau - \tau_{\min})/(\tau_{\max} - \tau_{\min})$ as a function of the radial distance from the centre of Saturn. The minimum and the maximum correspond to $\tau_{\min}^{\text{II}} = 1.1108$ and $\tau_{\max}^{\text{II}} = 3.5$, respectively.

FIG. 2 The optical-depth profiles normalized as in Fig. 1b. The corresponding τ -values are: $\tau_{\min}^{\text{I}}=0.0$, $\tau_{\max}^{\text{I}}=2.0504$, $\tau_{\min}^{\text{II}}=0.7208$, $\tau_{\max}^{\text{II}}=3.5$, $\tau_{\min}^{\text{III}}=0.7952$, $\tau_{\max}^{\text{III}}=3.5$, $\tau_{\min}^{\text{IV}}=0.7877$, $\tau_{\max}^{\text{IV}}=3.318$, $\tau_{\min}^{\text{V}}=0.0$, $\tau_{\max}^{\text{V}}=1.479$. Scale divisions in r are 20 km.

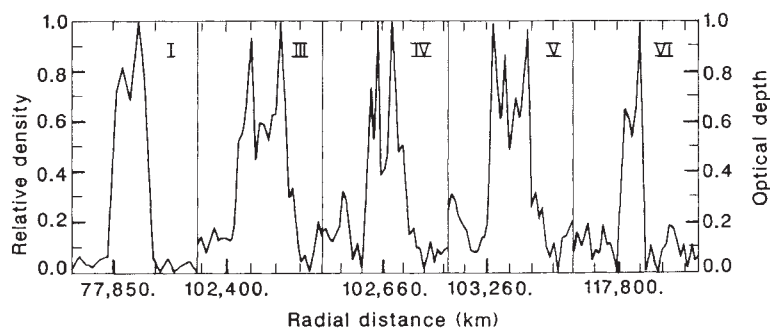
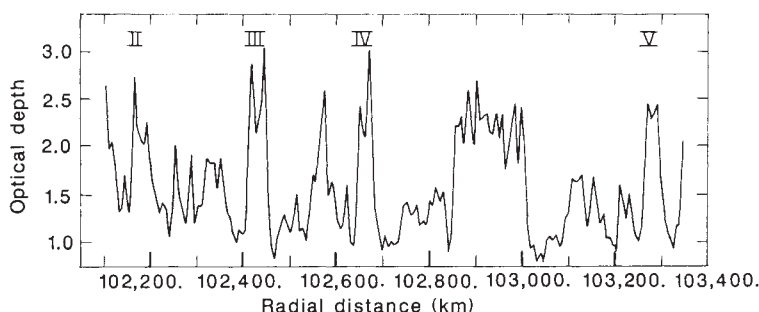


FIG. 3 Structure in the radial range around 1,300 km (middle of the B-ring), in which most of the scattering structures are concentrated. The units of optical depth are the original values obtained from the UVS measurements.

distance with respect to a_m , scaled by h) of the orbit of the moonlet ($b=0$), bounded by sharp density peaks and then by a pronounced drop in density for $1.0 < |b| < 1.2$; and (2) gaps that flank the inner ringlet for $1.2 < |b| < 4.0$.

The radial distance, $D \approx 2h$, between the remarkably stable peaks can be used to estimate an upper limit for the size of the embedded moonlet, if one assumes that it cannot exceed the size of its own Hills sphere ($2R_m < D$).

To search for moonlets in Saturn's rings, we inspected, independently several times (by eye), the Voyager UVS occultation data, consisting of 19,350 data sets which contain information on the optical depth τ , the connected radial distance r from Saturn, and an error quantity. We searched for events in the optical-depth profile that are similar to the results of our model calculations. These events were compared with their background surroundings using statistical methods to exclude any coincidental similarity of the data-point series. In these significance calculations, all marked structures other than the event itself were removed to obtain a background having a nearly normal distribution in τ . The complete scattering structures (density peaks and gaps) were judged by Fisher's F -test, and the significance of the density peaks was calculated with a modified t -test. The most significant examples of scattering structures found in the UVS data are shown in Figs 1b–3. The results of the tests and the distance D between the density peaks are listed in Table 1.

The test results eliminate with high probability a random origin of the significant structures (except for the F -test of

feature II, although this is nevertheless very similar to the model profile (Fig. 1)). The t -values in particular ensure a high confidence level of all density peaks; this is consistent with our long-term calculations, which indicate that the peaks are highly stable. Because the density contrast of the gaps at $|b| \approx 4$ tends to vanish⁵, there are examples in which a gap cannot be observed (features I and VI).

It is remarkable that the scattering structures II–V are concentrated in a relatively small radial region of width 1,300 km (Fig. 3). This fact could possibly be explained by the lack of resonances in that region¹² and thus, as mentioned above, by an inefficiency of viscous diffusion¹⁰, which normally counteracts all density gradients.

Thus we argue that moonlets should exist in planetary rings. The six examples presented of the UVS optical-depth profiles agree with high statistical significance with the calculated density distributions caused by the influence of a moonlet. A random origin of these examples is most improbable. In addition to these structures, we have found about 100 structures with smaller radial scales ($D \approx 7$ km). They also show features that are similar to the model profile, but the resolution of the UVS data does not allow a robust statistical confirmation. If most of these small-scale structures were caused by moonlets, this would be consistent with the power-law size distribution of moonlets¹³, $n(R_m) \propto R_m^{-q}$ (where $q \approx 5$ –6 for $R_m > 5$ m). The photopolarimeter occultation data, which have a higher resolution of 100–200 m, should be investigated in future to obtain further information about the size distribution of moonlets. □

TABLE 1 Distance between peaks, and statistical tests

Event	I	II	III	IV	V	VI
D (km)*	7.0	30.7	23.9	13.6	30.7	13.1
F -test	—	1.74	3.03	3.10	3.10	—
$(F_{cr})^\dagger$	—	(1.79)	(1.69)	(1.69)	(1.79)	—
t -test	7.14	7.64	8.84	8.98	9.51	4.56
$(t_{cr})^\dagger$	(3.37)	(3.32)	(3.32)	(3.32)	(3.32)	(3.37)

* Distances between peaks that are twice the Hill-sphere radius h of the corresponding moonlets.

$^\dagger F_{cr}$ and t_{cr} denote, respectively, the critical values $F_{0.01; N_b-1, N_e-1}$ and $t_{0.001; N_b+N_e-2}$ according to confidence levels of 99% and 99.9% (N_b and N_e are the degrees of freedom of the background and the event, respectively). Thus, the probability of random origin of all events is smaller than 1%.

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Chemical waves on spherical surfaces

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THE concentric-circular and spiral patterns exhibited by the Belousov-Zhabotinsky (BZ) reaction in thin films of solution are representative of spatiotemporal behaviour in a two-dimensional, planar excitable medium¹⁻⁶. Here we report BZ chemical waves propagating on the two-dimensional surface of a sphere. A wave on the surface of a single cation-exchange bead, loaded with ferroin and bathed in BZ reaction mixture containing no catalyst, develops to form a rotating spiral. Unlike spiral waves in thin films of solution, which typically wind out to connect with a twin rotating in the opposite direction, these waves rotate from pole to pole in a single direction. The spiral winds outward from a meandering source at one pole, crosses the equator, and undergoes self-annihilation as it winds into itself at the other pole. This behaviour, which is not possible in a two-dimensional planar configuration, arises from qualitative (negative to positive) and quantitative changes in wavefront curvature as the wave traverses the spherical surface. These observations of a single spiral wave contrast with theoretical predictions^{7,8} of counter-rotating spirals in this geometry.

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In an earlier study⁹, the redox indicator ferroin was loaded on cation-exchange resin in order to examine chemical-wave behaviour in an immobilized-catalyst BZ system. Pattern formation was studied in a 0.5-mm-thick layer of ferroin-loaded resin beads covered with a 1.6-cm-thick layer of reaction mixture containing no catalyst. A transition from regular to irregular patterns was found on increasing the concentration of either BrO_3^- or H_2SO_4 . Bead diameters in these experiments ranged from 38 μm to 75 μm , and individual beads appeared to be uniformly red or blue as a wave propagated through the bead layer. Similar experiments with much larger resin beads (diameter 0.5–1.4 mm) showed that wave patterns may develop on a single bead.

In our experiments, wave activity was allowed to develop in a collection of beads immersed in a bath of reactants, with some beads in physical contact and others almost touching. The initial wave behaviour is complex; the chemical environment surrounding a particular bead in the collection is not uniform, because of the wave activity on neighbouring beads. Apparently, a pair of counter-rotating spiral waves appear at first; if allowed to develop, one spiral rotor overtakes, and eventually 'consumes' the other, because their rotational frequencies differ as a result of the differing chemical environments. This process therefore results in single-rotor waves. Beads with interesting behaviour are mechanically moved from the collection to an isolated region in the petri dish to ensure a chemical environment that is as uniform as possible. Figure 1 shows a time-lapse sequence of a spiral wave propagating on the surface of a bead. Once isolated, such spirals are quite robust and seem to be undisturbed when the bead is moved. The behaviour also seems to be unaffected by orientation: the bead in Fig. 1 was mechanically reorientated

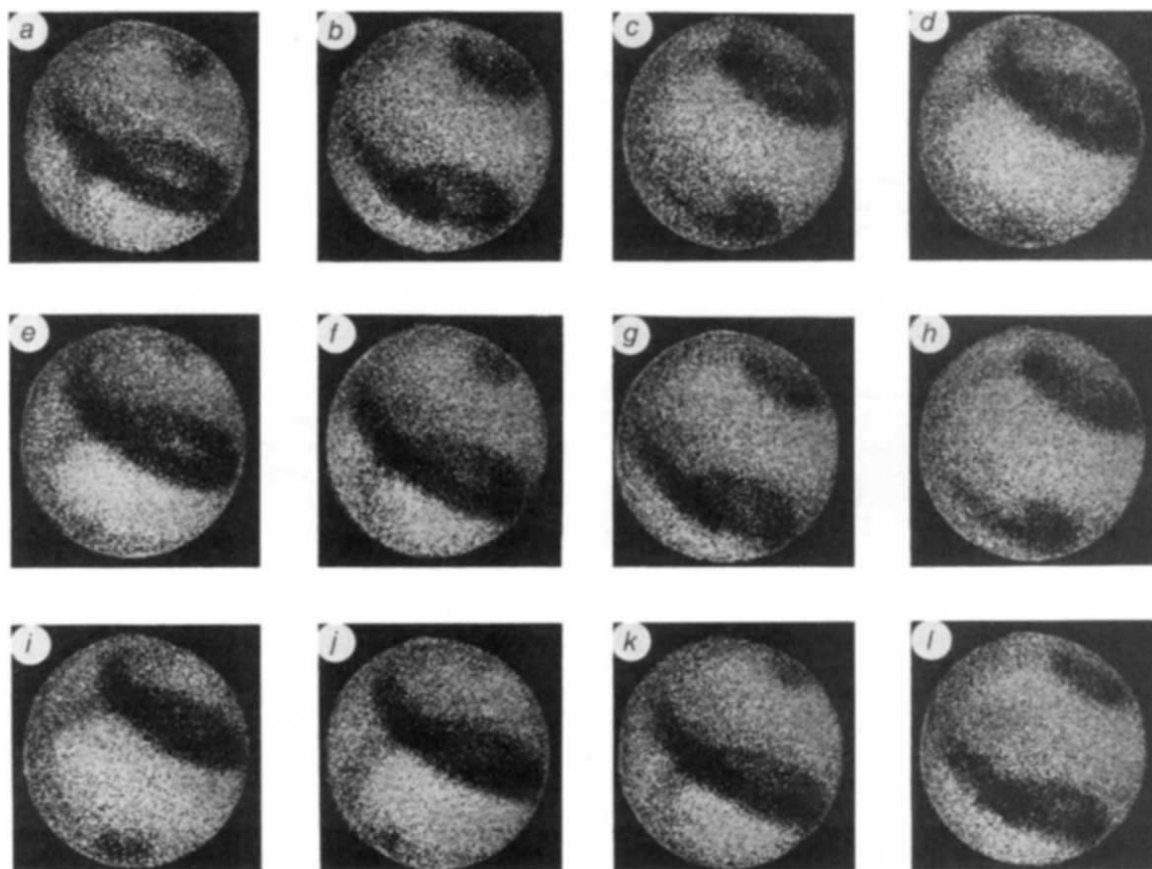


FIG. 1 Sequence of difference images, taken at 12.8-s intervals, of a spiral wave on the surface of a spherical cation-exchange bead of 1.38-mm diameter (Bio-Rad AG 50W-X4). The primary images are composed of 485×496 pixels, each digitized to 256 grey levels. The bead is illuminated by light from a tungsten source passing through a 10-nm bandpass filter

centred at 440 nm. The dark band represents an increase in grey level due to the conversion of ferroin to ferriin in the wavefront. The loaded bead (2.0×10^{-5} mol ferroin per g resin) is bathed in a 1.6-cm layer of reaction mixture maintained at 25.0 °C. Solution composition: $[\text{KBrO}_3] = 0.300 \text{ M}$, $[\text{H}_2\text{SO}_4] = 0.250 \text{ M}$, $[\text{malonic acid}] = 0.0250 \text{ M}$.

several times, and the time-lapse images showed only different orientations of the same spiral wave. Beads with counter-rotating spirals were also isolated. These beads exhibited very complex wave behaviour, with the two rotors apparently battling for limited space. Because the chemical environment is not precisely the same at each rotor, even when the beads are isolated, counter-rotating spiral waves should always eventually evolve to single-rotor waves, as described above. They may also undergo annihilation if the rotors collide.

To visualize the wave in Fig. 1, digital images taken at 12.8-s intervals were subtracted. The width of the dark band corresponds to the distance moved by the wave during the interval between the primary images. The wave is visible on both the front and back sides of the translucent bead, being slightly darker when in front. In Fig. 1a, the spiral tip (source) is just to the right of the 'north pole'. The wave winds to the left behind the pole and emerges in front of the bead at about the 10 o'clock position. It then crosses to about 4 o'clock, where it loops behind. The location of the wave can best be seen by following the movement from frame to frame. The successive images show a wave with spiral-like geometry continually rotating around the bead. The period of rotation is 64.9 s, and one period almost coincides with the sequence of frames a-f, f-k, or any similar diagonal sequence in Fig. 1.

The tip at the north pole is much like the spiral tips in thin films of solution. Rather than rotating around a fairly stationary core⁶, however, it seems to undergo complex motion similar to that found by Winfree *et al.*¹⁰ in a computational study of a reaction-diffusion Oregonator model. The time resolution of our measurements was not sufficient to accurately track the motion, but examination of successive frames suggests that the core, of about 0.1 mm in diameter, shifts by about a quarter of its radius for each rotation of the spiral.

Figure 2 shows oscillations in grey level as a function of time for a small region on the bead in Fig. 1. The first and second maxima in the apparent complex oscillations at the beginning of the time series result from the wave on the back and front sides of the bead, respectively. The wave crosses the sampling point in front and back at slightly different times each rotation, becoming superimposed in the second half of the time series. This lack of synchronization indicates that the oscillations are complex, consistent with the observation that the source tip at the north pole exhibits significant meandering.

The images in Fig. 1 indicate that the reaction-diffusion wave

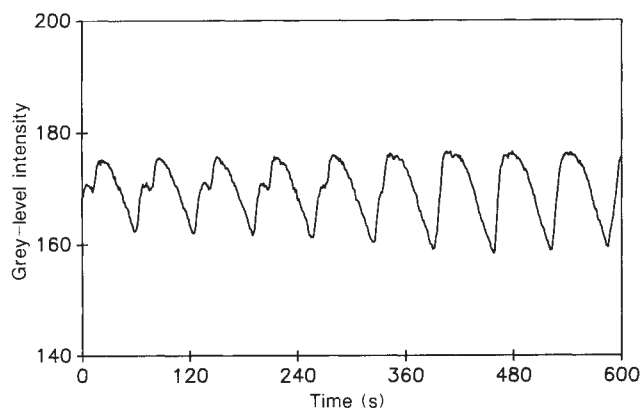


FIG. 2 Grey level, representing transmitted light (at 440 nm), as a function of time, measured for the bead in Fig. 1. The grey-level values represent averages over 1.0-s intervals from a 5×5 grid of pixels. The sampling grid represents approximately $18 \times 24 \mu\text{m}$. Average period $T = 64.9$ s. The apparent velocity of the wave in Fig. 1, given by the bead circumference divided by the period T , is about 4.0 mm min^{-1} . Figure 1 shows that the wave advances normal to the front at only about a quarter of this velocity. The velocity of waves in thin films of solution with the same concentrations of BrO_3^- and H_2SO_4 is expected¹² to be about 6.8 mm min^{-1} .

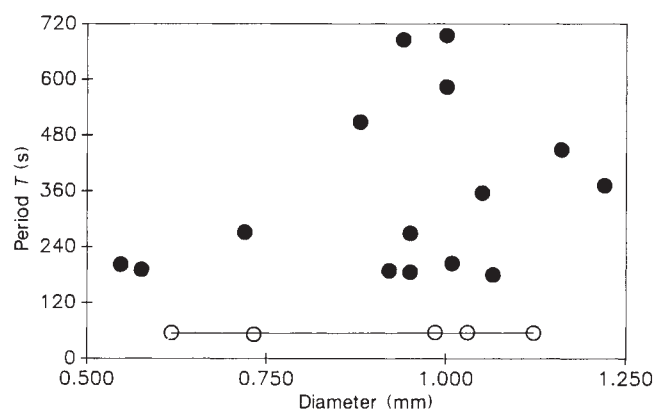


FIG. 3 Period T as a function of bead diameter for circular waves (●) and spiral waves (○). Period is defined as the average time between large-amplitude oscillations in measurements such as those in Fig. 2, rather than the (unknown) period of complex oscillation. Conditions and concentrations are the same as in Fig. 1, except that $[\text{KBrO}_3] = 0.375 \text{ M}$ for the circular waves and $[\text{KBrO}_3] = 0.250 \text{ M}$ for the spiral waves.

is indeed confined to the surface of the spherical bead. The net negative charge of cation-exchange resin prevents negative ions such as BrO_3^- and Br^- from appreciably penetrating the resin matrix⁹. These anionic species are essential components of the BZ reaction, so that significant reaction cannot occur within the resin matrix.

Although spiral waves like that in Fig. 1 were observed on a number of beads, other types of behaviour were also found. Symmetrically circular waves with wavelengths exceeding the bead diameter were found at higher BrO_3^- concentrations. Figure 3 shows period of oscillation as a function of bead diameter for spiral and circular waves. The varying period of the circular waves and the constant period of the spiral waves resemble the behaviour of 'target' and spiral patterns in thin films of solution. That the period of the spiral waves does not vary appreciably with bead diameter indicates that the rotation of the source tip is apparently little affected by the curvature of the bead surface. As in thin films of solution⁴, the rotational period of the spiral tip is determined by the composition of the medium, and this period, in turn, determines the period at any given point.

Theoretical studies of spiral waves propagating on a spherical surface have been made by Grindrod and Gomati⁷ and Brazhnik *et al.*⁸. Their analyses predict that counter-rotating spirals should develop at each pole of the sphere. These waves are symmetrical with respect to a plane of reflection at the equator. Single-rotor waves such as that in Fig. 1 lack this symmetry with respect to the equatorial plane. The wave winds outward from the north pole with decreasing negative curvature until it reaches the equator, beyond which it propagates with increasing positive curvature as it approaches the south pole. The dependence of velocity on wavefront curvature¹¹ predicts that the velocity increases as the wave winds out to the equator and then further increases as it winds in from the equator to the south pole. The spherical geometry forces the wave to wind into itself. As shown in Fig. 1, the self-annihilation at the south pole occurs in a periodic manner, with a full turn of the wave disappearing on each oscillation.

The complex temporal oscillations shown in Fig. 2 are due to meandering of the source tip. Depending on the dynamics of the meandering, quasiperiodic oscillations are also possible. The temporal behaviour of counter-rotating spirals may be even more complex if the meandering of each rotor is out of phase. Two or more beads in contact, each with single- or double-rotor spiral waves, exhibit a wide range of dynamic behaviour, which may include deterministic chaos. Our study of such complex behaviour will be reported elsewhere. □

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Fractality of sooty smoke: implications for the severity of nuclear winter

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IT is now recognized that the sooty fraction of the smoke produced by fires in the wake of a nuclear exchange is a critical factor in determining climatic effects^{1–3}. Sooty smoke particles occur as chained aggregates of small spherules which are fractal with a dimension of 1.7–1.9. According to a recently developed mean-field theory⁴ for the optics of such fractal clusters, their short-wavelength absorption and scattering characteristics should differ fundamentally from those of spheres. Here I present the results of simulations of scattering from computer-generated fractal clusters which confirm that the theory is appropriate to fractal soot. The theory indicates that soot absorptivity is insensitive to particle size and hence that coagulation in a soot aerosol affects only slightly its optical depth. Studies of the climate effects of nuclear war that model sooty smoke as coalescing spheres are therefore likely to underestimate the severity of 'nuclear winter'.

Sooty smoke is formed during the non-flaming combustion of oils, plastics and construction materials. It absorbs sunlight strongly; at visible wavelengths its specific absorption is typically 8–10 m² g⁻¹. It is estimated that sooty smoke provides about half the initial optical depth in a post-nuclear-war atmosphere⁵. The remainder would be provided by 'oily' smokes arising from the combustion of wood and vegetation, which are lower in elemental carbon content and very different in their morphology and ageing characteristics. This paper is concerned with sooty smoke only.

The opacity of the post-nuclear-war atmosphere alters as smoke aerosol is removed (principally through precipitation scavenging) and as the smoke-particle size distribution increases through coagulation in the dense plumes⁶. In the most comprehensive recent studies of nuclear-winter effects, smoke, both sooty and oily, is modelled as a distribution of spheres which, upon coagulation, merge into larger spheres. Smoke optical properties are derived from Mie theory. Coagulation is incorporated either by interactively adjusting the smoke size distribution as the smoke plume disperses^{7,8} or simply by assuming inflated initial smoke-particle sizes³. The effect of coagulation is found to be a reduction in the opacity of the smoke by up to two thirds^{3,7,8}.

Electron micrographs (Fig. 1) reveal that soot particles in fact occur as tenuous agglomerates whose open structure is not well modelled as spheres. Indeed, the observation that soot absorptivity is insensitive to degree of coagulation (N. P. Bryner, unpublished results) conflicts with the result of Mie theory for spheres.

An alternative model is provided by fractal geometry⁹. A fractal cluster possesses a statistical self-similarity (it looks more or less the same at any magnification) and is characterized by a fractal dimension D ($1 < D < 3$) which relates the mass M of the cluster to its characteristic radius R through $M \propto R^D$. The lower the fractal dimension, the more open the structure it describes. Sooty smoke particles are approximately fractal with a dimension of 1.7 to 1.9 (refs 10, 11).

A theoretical approach to the optics of fractal clusters was introduced recently by Berry and Percival⁴. In their formulation, an aggregate is modelled as a cluster of N identical spherules of radius much smaller than the wavelength of light, each of which scatters like a dipole. They make the (mean-field) approximation that the field at each spherule is influenced by multiple scattering to exactly the same extent. Then the electric field scattered by a cluster may be expressed in terms of the number of spherules, the spherule size, their refractive index and the cluster geometry. If the cluster is a fractal with $D < 2$ then the mean contribution to multiple scattering per spherule saturates in the large-cluster limit, so that the scattering and absorption efficiencies of large clusters tend to constants. If $D > 2$, multiple scattering per spherule increases as $N^{1-2/D}$, causing scattering and absorption efficiencies ultimately to decay as negative powers of N , although more slowly than in the geometric limit for a sphere. The optical distinction is sensible: whereas the shadow of a fractal cluster with $D > 2$ is compact and geometrically opaque, that of a fractal with $D < 2$ is still fractal and so geometrically transparent.

Because the mean-field approximation may fail when fluctuations in the electric field within a cluster are large, I have tested the theory using simulations of light scattering from a range of fractal clusters. Clusters representative of the two optical regimes were generated numerically by the mechanisms of particle-cluster diffusion-limited aggregation (DLA)¹² (which produces clusters of $D = 2.45 \pm 0.1$) and a hierarchical version of cluster-cluster DLA¹³ (from which $D = 1.80 \pm 0.05$). For fixed values of the cluster size N , refractive index and spherule size, the mean scattering and absorption cross-sections of large ensembles of clusters were computed in two ways: by exact solution of the multiple-scattering problem, and by making the mean-field approximation. Computer-time restrictions limited the cluster size to less than 50 spherules for these 'vector wave' simulations. To investigate larger clusters (up to 250 spherules), I have treated light as a scalar wave, thus simplifying the multiple-scattering problem. I varied the spherule refractive index from $1.4 + 0.3i$ to $2.0 + 1.0i$ and its radius from 5 to 25 nm to cover all values reported for soot, and used a visible incident wavelength. These

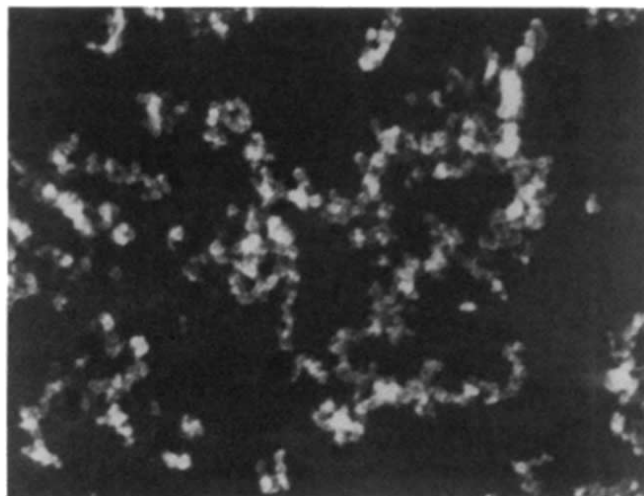


FIG. 1 Electron micrograph of a typical soot agglomerate (provided by E. J. Hardman of the Institute of Aerosol Science, University of Essex).

simulations are described in detail in a separate paper¹⁴.

In all cases the mean-field approximation agrees with the exact result to within 10% and (except in the scalar-light case) consistently underestimates both scattering and absorption. Assuming that the validity of the approximation extends to the large-cluster limit, our theory predicts that the absorptivity of large fractal clusters with refractive index, spherule radius and fractal dimension appropriate to soot settles at a level of 0.75 to 1.0 times the single-spherule value. The effect of clustering on the absorbing power of soot is therefore slight.

In Fig. 2 the scattering and absorption enhancements of fractal clusters of the particle-cluster-DLA type and the cluster-cluster-DLA type, as calculated from the mean-field theory, are plotted as a function of N and are compared with those of a sphere of equivalent volume. The scattering or absorption enhancement is the ensemble-average cross-section for a cluster divided by N times the single-spherule cross-section. Dimensionality is unimportant for small clusters; spheres absorb and scatter most strongly at intermediate cluster sizes (the Mie region) but fractal clusters of $D < 2$ are by far the strongest scatterers and absorbers in the geometric limit. Their absorption is more similar to that of a single spherule—whose absorption enhancement is constant at unity—than to that of the equivalent sphere.

This fundamental distinction between the limiting optics of spheres and of fractal clusters of $D < 2$ is reflected in the way in which the optical properties of a coagulating aerosol develop.

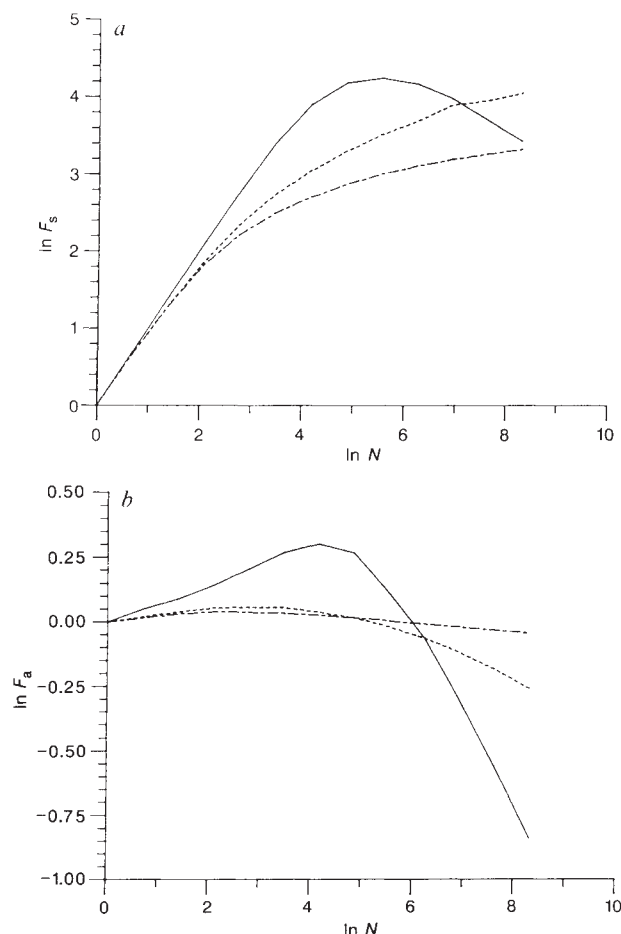


FIG. 2 *a*, Scattering (F_s) and *b*, absorption (F_a) cross-section enhancements as a function of number of spherules N for the equivalent-volume sphere (calculated by Mie theory) (full line) and for fractal clusters of $D=2.45$ (dashed line) and $D=1.80$ (chained line) (calculated by the mean-field theory). The spherule refractive index is $1.75+0.5i$ and the product of spherule radius and light wavenumber is 0.25.

To demonstrate this I have compared coagulation by spheres and by fractal clusters of the dimensionality of soot ($D=1.8$). Using a log-normal initial size distribution of mode radius $0.1\ \mu\text{m}$ and variance 2 (representative of the primary spherules that compose sooty smoke¹⁵) and an initial smoke mass concentration of $0.07\ \text{g cm}^{-3}$ (typical of nuclear-war scenarios), I have traced the evolution of the smoke size distribution by numerically integrating the Smoluchowski coagulation equation for spherical and fractal particles¹⁶. The evolving optical properties were computed using Mie theory for the spheres and the mean-field theory for the fractals.

In Fig. 3 the specific scattering and absorption cross-sections at a wavelength of 550 nm and refractive index of $2.0+1.0i$ are plotted against time. (Timescales are rather shorter than for nuclear-war conditions because dilution of the plume is neglected. This is not important here, where fractals and spheres are compared only at the same relative stage of coagulation.) Whereas, for advanced coagulation, scattering by fractals and spheres is comparable, fractals soon become the stronger absorbers (specifically, for equivalent-sphere mode radii $\geq 0.1\ \mu\text{m}$). The absorptivity of the fractal aerosol is remarkably insensitive to coagulation and even to the spherule radius, and to a good approximation (within 15%) it is given by the absorptivity of the primary spherule. Such behaviour for soot is supported by experiment (N. P. Bryner, unpublished results). When the particles have reached what Penner and Porch⁷ consider to be their

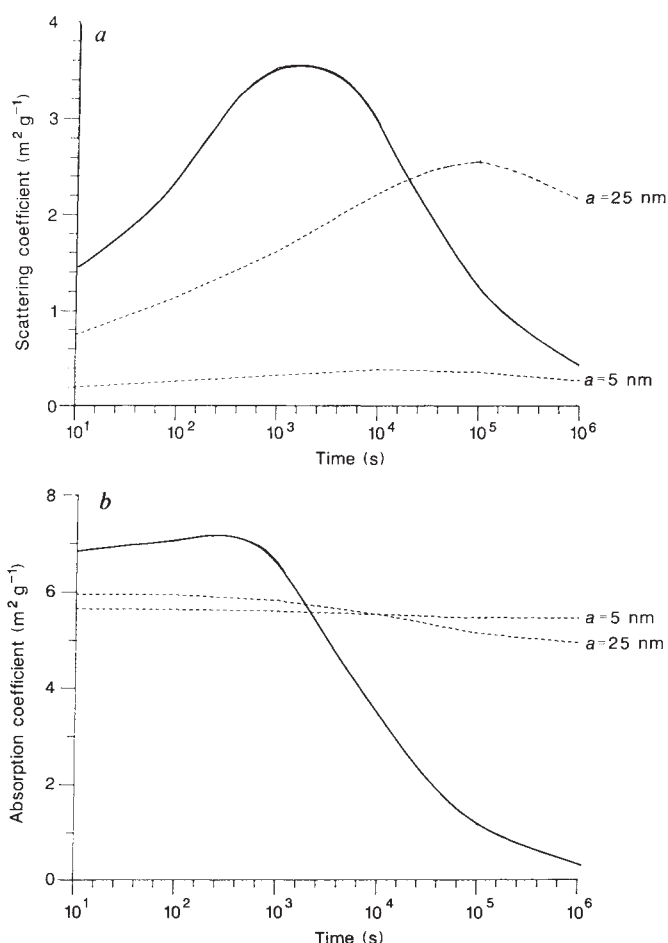


FIG. 3 *a*, Scattering and *b*, absorption cross-sections per unit mass as a function of time for coagulating aerosols of fractal particles (dashed lines) and spheres (full line). The spherule refractive index is $2.0+1.0i$ and the wavelength of light is 550 nm. Limits to the value of the spherule radius a are indicated.

final size distribution (a mode radius of about $0.4\text{ }\mu\text{m}$) the fractal aerosol absorbs more strongly than equivalent spheres by a factor of 4 to 5.

Soot is the key absorber in the post-nuclear-war atmosphere, and because soot particles occur not as spheres but as fractal aggregates with a dimension less than 2, a realistic assessment of nuclear-winter effects requires that the optical consequences of the fractality of soot be taken into account. The central question is how much of the smoke in a post-nuclear-war atmosphere would exist as fractal clusters.

Sooty smoke initially provides about half of the post-war optical depth⁵, but ageing may change this proportion. Upon contact with water vapour some smoke particles will act as nuclei for cloud condensation (CCN). Sooty smoke particles may collapse under such cloud-processing into compact structures more appropriately modelled as spheres (G. W. Mulholland, unpublished results). Although the fraction of soot particles likely to be processed in this way is not known—and so far soot appears reluctant to form CCNs¹⁷—the effect will be to reduce the relative proportion of fractal smoke. On the other hand, oily smoke particles form CCNs much more readily than does sooty smoke¹⁷ and so are more likely to rain out; and fractals fall more slowly under gravity, on account of the greater atmospheric drag¹⁸. Both of these effects will enhance the proportion of fractal soot in the smoke, in contrast to the cloud-processing effect.

The results presented above indicate that the absorptivity of fractal sooty smoke is underestimated whenever it is modelled as a distribution of spheres whose mode radius exceeds $0.1\text{ }\mu\text{m}$. Recent studies of the climatic effects of nuclear war are based on spherical smoke particles of mode radii from 0.2 to $0.4\text{ }\mu\text{m}$ (refs 3, 7). According to the study of Ghan *et al.*³, 'uncoagulated' smoke (spheres of mode radius $0.1\text{ }\mu\text{m}$) causes a fall in temperature in the mid-Northern hemisphere after 20–30 days that is some 5 – 10°C greater than that caused by the 'coagulated' smoke (spheres of mode radius $0.4\text{ }\mu\text{m}$) of their baseline study. Fractal smoke absorbs about as strongly as their 'uncoagulated' smoke. Therefore, unless the collapse resulting from cloud-processing is complete, so that none of the smoke produced remains fractal, their baseline study underestimates the severity of nuclear winter.

If it is valid to assume that soot provides half the optical depth, and we incorporate fractality approximately by setting soot absorptivity as constant at its measured value, the short-term effect of soot fractality is to make the nuclear winter colder by several (up to 5) degrees. Its long-term effect could be a doubling or trebling in the opacity of the residual smoke layer that is believed to stabilize in the upper atmosphere. Both effects are very significant in biological and climatic terms. They warrant further investigation of the morphology and cloud-nucleating capacity of smoke, and a serious treatment of smoke fractality in nuclear-winter simulations. □

The convective liquidus in a solidifying magma chamber: a fluid dynamic investigation

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CRYSTALLIZATION at the margins of magma chambers induces strong viscosity variations¹, and recent studies^{2–4} have shown how such crystallization affects the vigour of convection. Here we report an experimental study of the solidification of a layer of paraffin cooled from above. When the fluid is superheated, convection sets in at the beginning of cooling but rapidly decreases in intensity. Once the layer has lost its superheat, convection ceases and further cooling is by conduction. The interior temperature then remains constant until encountered by the upper crust. We define the convective liquidus as the temperature threshold below which convection is very weak or non-existent. In natural basaltic systems, the convective liquidus, although strictly unknown, may be very close to the true liquidus. Rapid convection may therefore not be a dominant process during the crystallization of many magma chambers; instead, convection is part of an overall intimate balance between phase equilibria, crystal growth and heat transfer.

It is essential in the experimental study of the interaction between crystallization and convection in magma chambers to choose an analogue fluid that has a melting range and no eutectic. The fluid that we used here is a mixture of pure paraffins (40% *n*-pentadecane $\text{C}_{15}\text{H}_{32}$ and 60% *n*-nonadecane $\text{C}_{19}\text{H}_{40}$), that forms a binary solid-solution in the system $\text{C}_{15}\text{H}_{32}$ – $\text{C}_{19}\text{H}_{40}$ (ref. 5). It has a liquidus close to 22.6°C and a solidus ~ 5 – 6°C lower; the physical properties⁶ are given in Table 1. The residual liquid is lighter and the crystals denser than the initial liquid. Density changes due to compositional effects over the melting range are $\leq 0.005\text{ g cm}^{-3}$. Our study of the viscosity in the melting range suggests that it does not vary by more than a factor of ten until the fluid becomes non-newtonian at ~ 1 – 2°C below its liquidus, and then the viscosity increases precipitously.

We used a $20\times 20\times 20\text{ cm}$ plexiglass tank with 2-cm-thick walls and bottom. The upper boundary is a 2.55-cm-thick metal plate in which water circulates under thermostatic temperature control. A comb of seven thermocouples attached to a vertical plexiglass rod in the middle of the tank provides a temperature profile normal to the roof. The calibrated thermocouples have an accuracy of $\pm 0.2^\circ\text{C}$. The initial temperature, T_i , in the fluid is uniform and slightly above the liquidus temperature T_L . At time $t=0$, the metal plate is switched to the cold bath and its temperature decreases rapidly within 4 min to $\sim 90\%$ of the final temperature T_f . This approximates instantaneous cooling.

All the experiments performed exhibit a similar behaviour which we describe qualitatively for the experiment 45 ($T_i = 24.2^\circ\text{C}$, $T_f = 17.4^\circ\text{C}$). As cooling proceeds, the temperature of the fluid in contact with the metal plate drops, allowing the formation of a thin solid crust ($< 1\text{ mm}$) as well as the develop-

TABLE 1 Physical properties of the paraffin mixture

Quantity	Symbol	Value
Specific heat (35°C)	c_p	$2\times 10^3\text{ J kg}^{-1}\text{ }^\circ\text{C}^{-1}$
Density (35°C)	ρ	$0.8\times 10^3\text{ kg m}^{-3}$
Thermal diffusivity	κ	$10^{-7}\text{ m}^2\text{ s}^{-1}$
Thermal expansion	α	$9\times 10^{-4}\text{ K}^{-1}$
Kinematic viscosity (35°C)	ν	$4\times 10^{-6}\text{ m}^2\text{ s}^{-1}$
Temperature of fusion	T_L	22.6°C
Melting range	$T_L - T_s$	$\sim 5^\circ\text{C}$
Heat of fusion	L_f	$1.7\times 10^5\text{ J kg}^{-1}$
Prandtl number	Pr	40

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ment of the first instability at time $t \approx 1$ min. Instabilities always set in below the crust, but appear not to carry crystals deeper into the fluid. The interface between the growing crust and the liquid is planar and crystals are short (~ 1 mm in length) and hair-like (dendritic). At the beginning of cooling, strong convective plumes with maximum velocities of $\sim 2 \text{ mm s}^{-1}$ can be followed until they reach the bottom of the tank (Fig. 1a). Although calculation of the Rayleigh number, $Ra = g\alpha\Delta T d^3 / \kappa\nu$ (where d is the total depth of the tank, κ is the thermal diffusivity, ν is the kinematic viscosity, α is the coefficient of expansion and ΔT is the temperature differential), yields $Ra = 3 \times 10^8$ for $\Delta T = 1.6^\circ\text{C}$, the convection is three-dimensional and unsteady but not turbulent (that is, there are no small-scale eddies and the Reynolds number is low, ~ 100). After two hours, convection decreases severely; maximum plume velocities are $\sim 0.5 \text{ mm s}^{-1}$. Plume velocities decrease to such an extent that noticeable motion is restricted to progressively higher levels (Fig. 1b, c). After three hours, the fluid has lost nearly all of its superheat and the interior temperature is everywhere very close to the liquidus. Convection is then very sluggish (Fig. 1c). Note that the thickness of the crust is only ~ 2 cm when noticeable convection ceases whereas it takes about a further four days to crystallize the whole tank. At this stage, the fluid below the crust contains no crystals.

Temperature profiles as a function of time are shown in Fig. 2. Note that a temperature gradient is observed only in the crust and in the thin upper boundary layer. The interior temperature decreases uniformly towards the liquidus as cooling proceeds and the upper crust grows downward. As buoyancy vanishes, convection ceases. The interior then remains at the liquidus and temperature at a given depth remains constant until reached by the downward-propagating crystallization front. No significant thermal stratification is evident at any time. The temperature at which convection becomes very weak or non-existent is defined here as the 'convective liquidus' and has been found to be equal

to the thermodynamic liquidus, within the accuracy of the measurements, regardless of the initial amount of superheat. This is the first critical feature of these experiments. The existence of a convective liquidus holds regardless of the cooling geometry, as we have also performed experiments on the cooling along a vertical side wall.

Figure 3a is a plot of temperature against time at different depths for experiment 47 which is similar to 45 but has a longer data set. Two stages can be distinguished: 'active' convection (stage I), where the interior temperature rapidly decreases to the liquidus, which is followed by stage II where cooling proceeds essentially by conduction and the interior temperature remains at the liquidus. The second critical point in these experiments is that the duration τ of stage I is very short compared with the total solidification time or the duration of stage II (Fig. 3a). The duration of stage I depends on the amount of superheat $\Delta T = T_i - T_L$ and the thickness of the tank. It can be calculated using the formula given in ref. 7:

$$\tau = \left(\frac{14.3 d^2}{\kappa} \right) Ra^{-1/3} \quad (1)$$

where the Rayleigh number is based on the whole tank depth d ; τ is equal to 124 min in the case of experiment 47 and represents $\sim 4\%$ of the total conductive solidification time (Fig. 3a). On the other hand, quiescent stage II cooling is closely approximated by conduction only, in agreement with other studies⁸. It is during stage II that the principal crystallization of the tank takes place. Finally, at times $t > 12$ h, crystals start to form on the bottom and the sidewalls, because of unavoidable wall and floor cooling. This produces some evolution of the liquid composition during the crystallization of the whole tank and may explain the departure of the interior temperature from the liquidus at depth $z = 9.8$ cm after $t = 14$ h (Fig. 3a).

Before extrapolating the results of the present experiments to

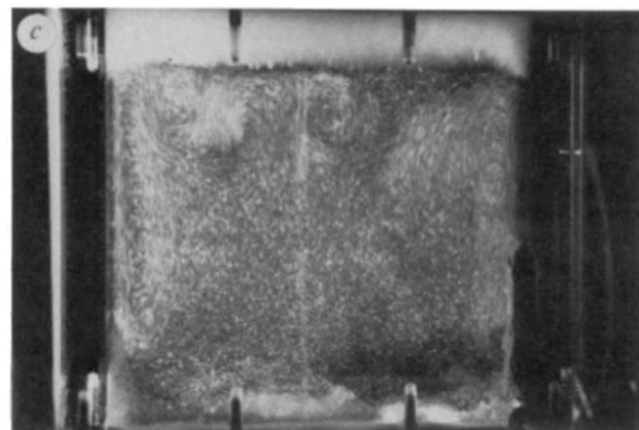
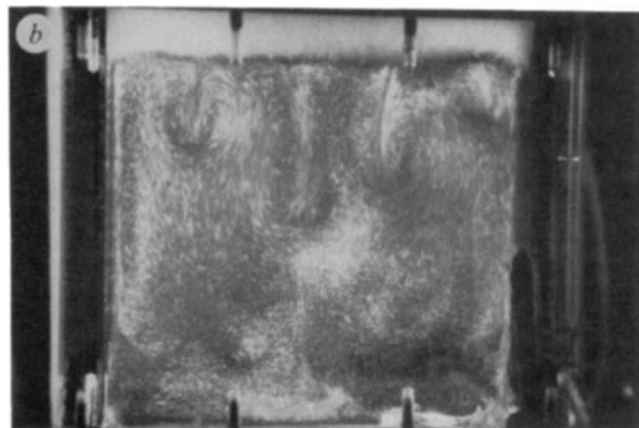
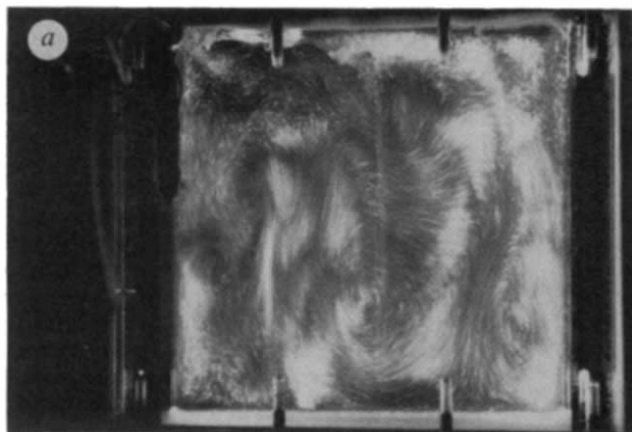


FIG. 1 Time sequence for the development of convection (in experiment 45). These pictures are time-lapse exposures of 5 s (a, b) and 10 s (c). The flow is visualized with small aluminium flakes ($< 100 \mu\text{m}$) illuminated from the side by a vertical sheet of light. These photographs indicate the strength and style of convection by comparing the streak length in a thermal plume with that of the ambient fluid. a, After 21 min 25 s, convection is vigorous and plumes can be followed until they reach the bottom of the tank. Note however that this is not a turbulent pattern. b, After 2 h 1 min 30 s and c, 2 h 46 min 30 s, convection severely decreases. Plume velocities decrease to such an extent that noticeable motion is restricted to progressively higher levels. Convection is always intermittent and the boundary layer breaks into convective plumes. Note also the small thickness of the crust when convection is dying (c).

natural systems, it is necessary to understand the structure of the unstable part of the boundary layer⁹⁻¹², where the viscosity changes as a result of the gradients of temperature, composition and crystal content. First, there might be some important compositional effects in the boundary layer. Note, however, that the chemical diffusivity, although strictly unknown, is usually much smaller than the thermal diffusivity, and that compositional effects do not become significant before the cessation of thermal convection (Fig. 3a). As cooling proceeds, compositional effects might become important in stifling convection, but this is also the case in magmas. In terms of the effect of crystal content, some crystals might be carried by convection into the interior, especially if the crystallization timescale is long relative to the timescale of convective instability². No crystals were observed in the unstable boundary layer, which may imply that crystallization is rapid compared with convective instability. This could, however, also be because nucleation is heterogeneous and the crystals form an interconnected dendritic network that cannot take part in the unstable boundary layer.

The paraffin properties are, however, different from those of magmas (Table 1) so how do our results relate to magmatic systems? For paraffin, the Prandtl number, Pr , is 40 and $Ra > 10^8$, so convection could possibly take a different form from that in magmas¹³, where Pr is larger. Nevertheless, no turbulence was ever observed. The Stefan number $\sigma = c_p \Delta T / L$ is > 0.1 but less than that in magmas, thus the heat released at the interface is affected only slightly during the propagation of heat through the crust¹⁴. However, a larger σ could be obtained by fixing T_f at a lower value, without changing the results. In magmas, the density changes arising from compositional effects are usually larger than thermal changes, unlike the paraffins used here. However, there is no chemical evolution without crystallization. Crystallization requires some cooling and it is essential to study the thermal regime. We therefore suggest that a similar behaviour could be observed in natural systems, where the residual liquid is light. Vigorous convection would seem possible until the magma reached a prohibitively high viscosity as a result of increasing crystallinity, which might occur after $\sim 50\%$ crystallization¹⁵. However, this critical crystallinity is bound to be dependent on the strain rate of convection. We need to discover the degree of crystallinity at which magmatic convection ceases.

Crucial information on this issue comes from the thermal history of Hawaiian lava lakes¹⁶⁻²¹, formed by eruptive filling

of pit craters 15–120 m deep. Phenocrysts in the initial magma settled out, leaving the body at its liquidus temperature, and the central temperature remained relatively close to the liquidus until crystallization fronts reached the centre. The shape and evolution of the natural vertical temperature profiles are strikingly similar to those observed in these experiments (compare our Fig. 2 with Figs 7 and 8 in ref. 20). Figure 3b represents the data for the estimated central temperature in the lava lakes

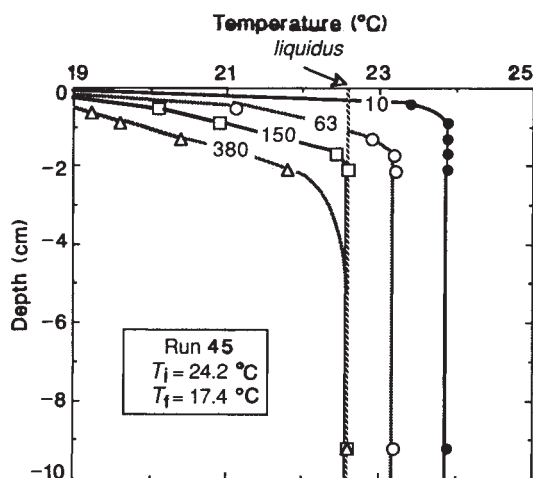


FIG. 2 Vertical profiles for the interior temperature at different times (experiment 45). Time is indicated in minutes along the curves. The thermal gradient is restricted to the solid crust and a thin boundary layer. Temperature is uniform in the interior. At a given depth in the interior, temperature decreases only to the liquidus (22.6 °C) and is then locked to this temperature until the crust encounters this point.

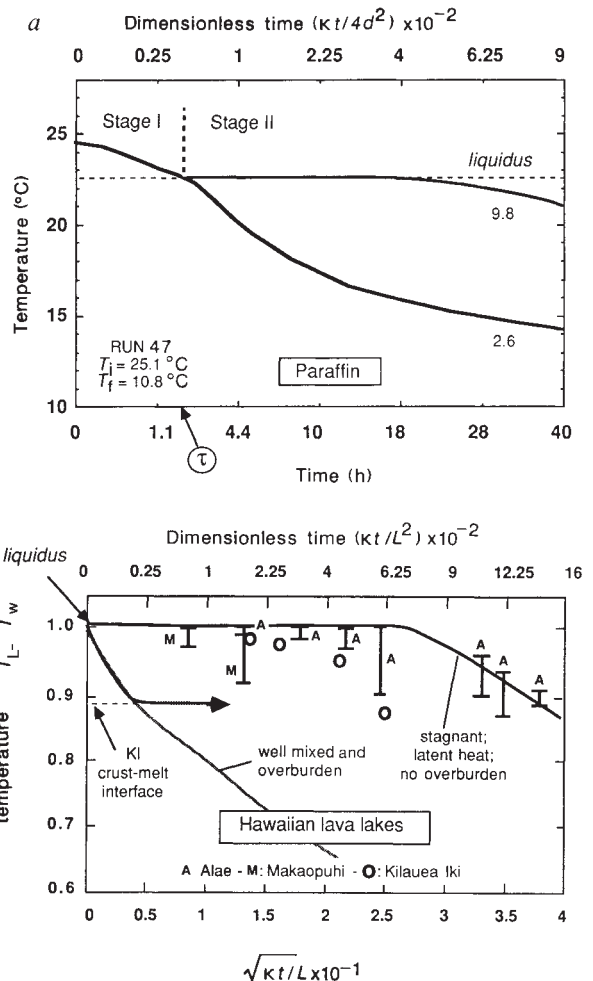


FIG. 3 a, Temperature versus time at different depths (indicated in cm under the curves) (experiment 47). The depth $z = 2.6$ cm is completely within the crust after $t = 2$ h, whereas $z = 9.8$ cm remains at the liquidus. The scale is linear in $\sqrt{t}$. Dimensionless time $t^* = \kappa t / 4d^2$ is also given as a scale for comparison with a body of thickness L cooled from above and below. Two stages can be distinguished. For $t^* < \tau$ or $t < 0.005$, cooling proceeds uniformly in the whole tank by convection (stage I). For larger times $t > \tau$, cooling proceeds mainly by conduction (stage II). Interior crystallization starts only when encountered by the growing crust. b, Variation in central temperature with dimensionless time $t^* = \kappa t / L^2$ during cooling from above and below of a layer of thickness L for two extreme cooling models. The first (upper curve), assumes the minimum rate of heat transfer (as calculated in ref. 4, assuming no convection, no overburden and taking into account the effect of latent heat). The second (lower curve) assumes the maximum rate of heat transfer (obtained for a perfectly well mixed model²⁹ where temperature decreases uniformly with time throughout the layer). The data and error bars correspond to estimated temperatures in the interior during solidification of Hawaiian lava lakes (refs 4, 16–20 and R. T. Helz, personal communication). Dimensionless times and temperatures are calculated assuming $\kappa = 10^{-6} \text{ m}^2 \text{ s}^{-1}$, $T_w = 0^\circ \text{C}$, $L = 13.7$ m and $T_L = 1,150^\circ \text{C}$ for Alae, $L = 83$ m and $T_L = 1,190^\circ \text{C}$ for Mākaopuhi, $L = 120$ m and $T_L = 1,200^\circ \text{C}$ for Kilauea Iki. Also indicated by the curve with the arrow is the thermal evolution of Kilauea Iki, assuming that rapid convection continues until reaching the temperature of the crust–melt interface for which crystallinity is $\sim 50\%$ (ref. 18).

as well as the temperature evolution calculated assuming two extreme cooling models (see Fig. 3 legend). The thermal histories of the lava lakes are closer to the conductive curve representing stagnant magma. Temperatures in Kilauea Iki depart from the ideal conduction model after $t^* \sim 0.0225$, which may be due to such observed processes as diapiric transfer of melt, crystal settling or gas exsolution¹⁸. However, there is no significant decrease of the interior temperature at early times, after the main degassing event ($t^* \approx 0.0045$; ref. 21 and M. Mangam, personal communication) or even after its complete cessation ($t^* \approx 0.013$). Comparison of the experimental (Fig. 3a) and the observed thermal (Fig. 3b) histories shows a remarkable similarity in terms of the temporal constancy of the interior temperature. This suggests the existence of a convective liquidus in the lava lake that is apparently near the actual magmatic liquidus itself. We suggest that it might not be the exsolution of gas that impedes convection¹⁷, but the advancement of the growing crust which partitions from the magma the buoyancy necessary for convection.

In a closed system, although convection may be vigorous at the beginning of cooling and may be able to carry crystals into the interior and evacuate the large amount of latent heat released by the crystallization, its intensity soon diminishes. Kinetic effects are important in cooling magmas, as has been stressed in numerical modelling²². This is confirmed by recent observations^{23,24} that crystallization of plagioclase in the Makaopuhi lava lake takes place at an undercooling of $<10^{-1}$ °C. The present experiments suggest that a thermal equilibrium will be achieved and the convective liquidus will be locked to the liquidus itself. They also show that, once the convective liquidus is reached, convective cooling may be negligible. This may also explain the results of studies^{4,25} that show that the solidification of lava lakes can be modelled by conductive cooling only, in contrast to previous experiments that do not involve a convective liquidus^{7,26,27} and calculations that assume turbulent convective cooling^{7,27,28}.

We therefore suggest that these results may hold for magma chambers evolving as closed systems, where the upper crust develops and the residual liquid is lighter than the original magma. In such chambers intruded at the liquidus, convection is apt to be sluggish and the interior temperature remains at the convective liquidus until being encountered by the growing crust. In magma chambers intruded with some initial superheat, the period of rapid convection will be short compared with the total solidification time (5 years compared with $\sim 3,000$ for a 1 km-deep basaltic chamber, intruded with a ΔT of 10 °C). Subsequently the interior temperature remains at this convective liquidus, which is close to the liquidus itself. This rapid loss of superheat may explain the well-known absence of superheated magmas in the near-surface environment. We suggest that, to a good approximation, the initial period of rapid convective cooling can be ignored and overall the solidification history of many magma chambers can be adequately modelled by conduction only. □

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Acoustic emissions and shear instabilities during phase transformations in Si and Ge at ultrahigh pressures

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ACOUSTIC emissions are commonly observed at low pressures as they are characteristic of brittle deformation^{1,2}. Most solids are ductile above a few gigapascals (GPa), and acoustic emissions have not been recorded from solids above 5 GPa. Here we describe acoustic emissions and shear instabilities that are associated with the β -Sn $\rightarrow$ simple-hexagonal phase transformation in Si and Ge to pressures as high as 70 GPa, well above the brittle-ductile transitions of both elements. We propose that the events are driven by rapid atomic motions across displacive phase transformations, and not by fracturing or cracking of the samples. These phenomena may be relevant to the processes that generate deep-focus seismicity in the Earth's mantle.

We compressed powdered Si and Ge (grain size 1–3 μm) without a pressure medium in a Mao-Bell diamond cell³ at room temperature. Less than 5% by volume of ruby powder (grain size $\leq 3 \mu\text{m}$) was placed at the interface between the sample and the upper diamond, and the pressure was measured with the ruby fluorescence technique⁴. We determined the shear stresses in each run by measuring pressure gradients across the sample⁵, and we observed the sample deformation visually with an optical microscope.

Previous studies have documented brittle deformation in Si and Ge to confining pressures of 5 GPa at room temperature⁶. When compressing samples of Si and Ge from zero pressure, we find that this brittle, stick-slip deformation appears as sudden shearing within small regions of the sample accompanied by rapid changes in the stress birefringence of the diamonds. These visual observations indicate that the brittle-ductile transition occurs at pressures close to the diamond $\rightarrow$ β -Sn phase transformation in Si and Ge at room temperature (~ 9 GPa; refs 7–9). This particular phase transformation involves a considerable volume change ($\Delta V/V > 0.17$), and is always associated with large amounts of ductile deformation distributed over the entire sample.

As we compress Si through the β -Sn $\rightarrow$ simple hexagonal transformation ($P \approx 16$ GPa; refs 7, 8), we observe sudden, brittle-like deformation at pressures well above the brittle-ductile transition. At the transformation pressure, the polycrystalline silicon produces large acoustic emissions that we can visually correlate with rapid displacements through the sample over lengths of $\sim 50 \mu\text{m}$ (Fig. 1). The abrupt nature of these instabilities resembles the brittle deformation that we observe at lower pressures, but it is unusual given the relatively low

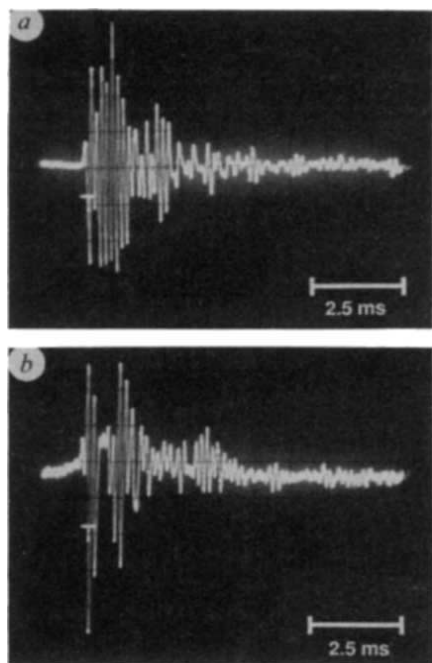


FIG. 1 Oscilloscope traces of the acoustic emissions from diamond-cell samples. The emissions were recorded with an acoustic microphone placed near the diamond cell. The Si emissions (a) were detected on compression at peak pressures of 20 GPa. The emissions from Ge (b) were recorded on decompression at peak pressures of 70 GPa.

shear stresses, $\sigma < 1$ GPa, relative to the confining pressure^{2,10}. Although the source regions are smaller than 10^{-14} m³, the emissions are clearly audible more than 1 m from the diamond cell. The acoustic emissions in Fig. 1 were recorded with an ordinary acoustic microphone (Aiwa, model CM-30).

With increasing pressure, the emissions and anomalous deformation continue while the pressures are near 16 GPa (Fig. 2). But when Si is compressed well above 16 GPa, the emissions cease and it deforms in a ductile fashion. On decompression, the emissions and deformation occur at ~ 11 GPa, and they are reversible on cycles of increasing and decreasing pressure. These results agree with the transformation pressures and hysteresis that are observed between the β -Sn $\rightarrow$ simple hexagonal structures in Si, 13.4–16.2 GPa on compression and 14.5–11.0 GPa on decompression as seen from X-ray diffraction at room temperature^{7,8}. We note that similar compression of a wide variety of materials fails to produce acoustic emissions^{5,11,12}; such

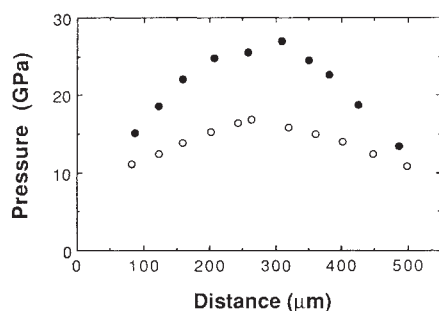


FIG. 2 Pressure profiles across a Si sample in the diamond cell. The upper and lower curves show the range of pressure over which acoustic emissions and shear instabilities are observed on compression. In general, the anomalous behaviour occurs when a portion of the sample is near the β -Sn $\rightarrow$ simple-hexagonal transition pressure (~ 16 GPa). The pressure gradients indicate that the shear stress in this sample is ~ 0.8 GPa.

behaviour is not a general property of solids at high pressures nor is it a feature of the diamond cell itself. Indeed, to our knowledge this is the first published report of acoustic emissions recorded from a sample in the diamond cell.

To obtain more information about these emissions, we attached a piezoelectric transducer directly to one of the diamond anvils. About 500 μ m in diameter, the transducer has a resonant frequency of 20 MHz and it is capable of detecting events with intensities that are well below the threshold of the microphone and human hearing. Qualitatively, the transducer shows that the emissions from the Si samples at the β -Sn $\rightarrow$ simple-hexagonal transition pressure have a wide range of intensities and durations (Fig. 3). Large events that are clearly audible saturate the transducer for longer than 1 ms, consistent with the event durations detected by the microphone in Fig. 1. Smaller events that are audible and can be detected by the microphone are well resolved by the transducer and have slightly shorter durations of ~ 0.6 ms (Fig. 3a). The transducer also detects events that are inaudible. These events can be visually correlated with deformation of the sample and they seem to have durations as short as 20 μ s (Fig. 3b).

In similar experiments on Ge, there are no audible emissions to pressures as high as 90 GPa, yet we repeatedly observe sudden (brittle-like) deformation and acoustic emissions on decompression at ~ 70 GPa (Fig. 1). (The different behaviour on increasing and decreasing pressure may not be significant as we have only used the microphone in these experiments.) The acoustic emissions cease as germanium is decompressed below 70 GPa. Hence their occurrence is consistent with the observed transition pressure of 75 GPa for the β -Sn $\rightarrow$ simple-hexagonal transition in Ge¹³.

At low pressures, similar acoustic emissions and rapid shearing can be associated with displacive phase transitions which exhibit local transformation rates near the acoustic velocity of

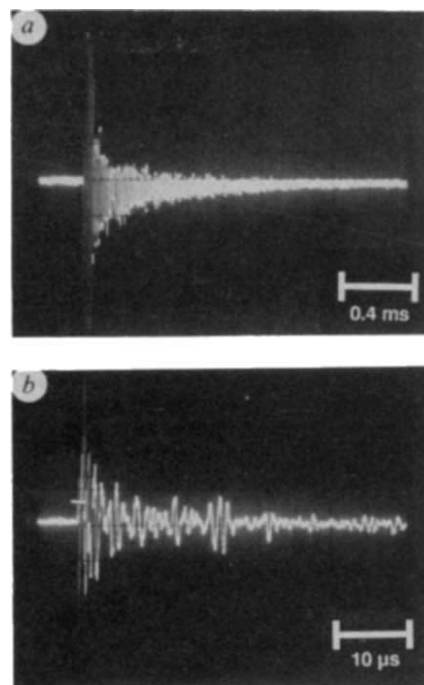


FIG. 3 Acoustic emissions from a Si sample in the diamond cell detected by a piezoelectric transducer mounted directly on one of the diamond anvils. The resonant frequency of the transducer is 20 MHz. The peak pressure in the sample is 19 GPa. Audible events have durations close to 1 ms (a). Inaudible events, visually correlated with displacements through to sample have shorter durations of ~ 20 μ s (b).

the sample¹⁴. In steels and shape-memory alloys, these mechanical anomalies are correlated with the growth of the new phase without any evidence of fracturing or cracking¹⁵⁻¹⁷. As the ΔV of these transitions are small, the emissions probably do not reflect sudden isotropic volume changes. Rather, they seem to be generated by the shear translations associated with the phase transformations. Previously, these phenomena have been documented only at low pressures ($P < 1$ GPa), yet they should not be inhibited by increasing pressure because frictional sliding is not involved. We propose that the acoustic emissions from Si and Ge are analogous to these emissions associated with low-pressure displacive transformations because the geometrical relations between the β -Sn and simple hexagonal structures are consistent with a displacive transition mechanism (see ref. 18, Fig. 4) and the volume changes across the transitions are small ($\Delta V/V < 0.017$; refs 7, 8, 13).

On the scale of an individual grain, the transformation probably occurs at the sound velocity, producing a discrete acoustic pulse. The acoustic velocities of Si and Ge ($> 4 \text{ km s}^{-1}$) imply that this pulse would traverse a sample in $\sim 10^{-7}$ s, yet we find that the emissions have durations as long as 10^{-3} s (Figs 1, 3). This difference in timescales (acoustic travel time versus the duration of each emission) cannot be explained by internal reflections of the emissions within our samples. Given the acoustic velocity of diamond ($> 10 \text{ km s}^{-1}$) and a conservative estimate of the reflection coefficients within the diamond cell (~ 0.9), an acoustic pulse should 'ring' for $< 10^{-5}$ s. (Observations using the transducer mounted on the diamonds confirm this estimate.)

Similar differences between the acoustic travel time and the duration of acoustic emissions are observed across displacive transitions at zero pressure¹⁶, and they can be explained as follows. When a transition involves shearing, even with a small ΔV , the transformation of a single grain significantly alters the surrounding stress field in a polycrystalline aggregate¹⁹. If this change in stress promotes further transformation in neighbouring grains, the phase transition can propagate over long distances by autocatalytic nucleation and growth²⁰. The transition moves sequentially from grain to grain, rather than nucleating homogeneously within the sample, and the velocity of the phase boundary can be far less than the acoustic velocity of the sample. Thus, we propose that the emissions in our experiments have long durations because they represent a continuous sequence of many discrete transformation events. Moreover, we infer that the wide variation in emission intensity and duration reflects a wide variation in the number of discrete events associated with each burst of transformation.

These results may have implications for understanding the occurrence of deep-focus earthquakes in the Earth's upper mantle. There have been numerous attempts to identify shear instabilities that could generate such seismicity at high pressures ($P > 10$ GPa), but laboratory experiments have not detected brittle deformation at pressures > 5 GPa (ref. 6). Unusual types

of failure have been described at low pressures^{6,21,22}, but they may not be operative at depth in the mantle because they all involve frictional sliding on cracks. Thus, our observations of acoustic emissions and shear instabilities associated with particular phase transformations in Si and Ge may be significant in providing the first experimental evidence for seismic phenomena at ultrahigh pressures, without fracturing and well beyond the brittle-ductile transitions of solids. $\square$

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Reassessment of comparative evidence for Hamilton and Zuk theory on the evolution of secondary sexual characters

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HAMILTON and Zuk¹ proposed that elaborate secondary sexual characters may have evolved because they act as cues in mate choice by revealing the health of a potential mate. From this theory, they derived the prediction that species which are more liable to parasitic infection should possess well-developed secondary sexual characters. They went on to demonstrate a positive association between prevalence of haematzoa and plumage brightness across species of North American passerine birds. Subsequent analyses by one of us² on the same colour data confirmed that the relationship with male brightness existed even when the effects of phylogenetic associations were taken into account. The relationship was also found across European passerines when behavioural and ecological correlates of colour and phylogeny were accounted for². Here, using independently derived plumage brightness scores, we question these conclusions.

The 114 North American species used by both Hamilton and Zuk¹ and A.F.R.² had been scored by Hamilton and Zuk for brightness "on a showiness scale of 1 to 6, with 1 being very dull and 6 very striking" (p. 385 of ref. 1). We asked each of six ornithologists to score the same species from pictures in a standard field guide³, using the same criterion. Our scorers had not seen the parasite data, they were not told why we were

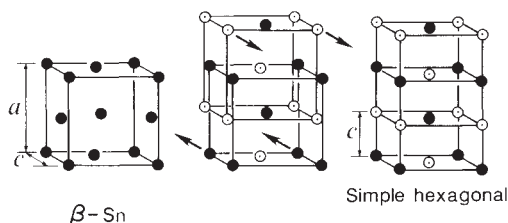


FIG. 4 Illustration of the geometrical relations between the β -Sn and simple hexagonal structures (after ref. 18). The tetragonal β -Sn structure (left) can be represented as two interpenetrating lattices offset by a distance of $c/4$ perpendicular to the a axis (centre). A simple shear of these two lattices in the c direction and a slight modification of the c/a ratio produces the simple hexagonal structure (right).

undertaking the exercise and they did not discuss their scoring with each other.

The colour scores of the species were highly correlated between ornithologists (mean Spearman rank correlation coefficient (r_s) = 0.76, range 0.61–0.88) and with the scores of Hamilton and Zuk (mean r_s = 0.74, range 0.55–0.82). The correlation across species between parasite prevalence (data sources and method of calculation given in ref. 2) and Hamilton and Zuk's brightness scores is highly significant (r_s = 0.26, P = 0.003). (We report two-tailed P -values throughout because we believe that the null hypothesis would have been rejected had a significant negative relationship been found.) The correlations between parasite prevalence and the scores of each of our respondents are always lower, and significant in only one case (r_s = 0.09, 0.13, 0.15, 0.15, 0.18, 0.22, P -values 0.33–0.02; mean correlation, r_s = 0.15, P = 0.10). The correlation between prevalence and the averages of the six new brightness scores for each species is 0.18, P = 0.06.

Correlations can arise, however, through confounding variables which are often associated with phylogeny^{4,5}. We repeated A.F.R.'s within-genera analysis which controls for phylogenetic correlates (details of the taxonomy used are given in ref. 2). There is a positive correlation between prevalence and the average of the six brightness scores in only 8 of the 19 genera containing two or more species (two-tailed binomial P = 0.81; mean intra-generic r_s = -0.01). There is no evidence of a consistent association within genera using the new scores, but there is when Hamilton and Zuk's scores are used². Looking within all taxa (by comparing species within genera, generic means—calculated as averages of constituent species values—within tribes, and tribal means within subfamilies, and so on) produces similar conclusions (positive correlations within 23 of 40 taxa, P = 0.42), suggesting that the weak interspecific correlation found using our scores is the result of phylogenetic associations. Indeed, the association arises at least in part because the Fringillidae (finches, buntings and tanagers) tend to be brighter and to have more parasites than other taxa. When the Fringillidae are excluded, the across-species relationship becomes negative (r_s = -0.05, n = 49, P = 0.71), and within the Fringillidae the interspecific relationship is not significant (r_s = 0.16, n = 65, P = 0.20).

How do our new scores differ from those of Hamilton and Zuk? One of the 114 species was given an average score more than one point higher than Hamilton and Zuk's, while 24 species were given a score of more than one point lower. These 24 species have a significantly higher parasite prevalence than the other 90 (Mann-Whitney U = 735, P = 0.01). Twelve of the 24 species are warblers (Parulini), and the rest are widely distributed taxonomically. Consequently, these species affect correlations within and across taxa. We can see no colour patterns that the 24 species have in common. There could be several reasons for the differences between Hamilton and Zuk's scores and those we obtained. For example, although five of our six scorers have each seen about half of the North American species in the wild, it is possible that Hamilton and Zuk were more familiar with the birds in the wild and could therefore better assess their showiness. Hamilton and Zuk's scores may include some aspect of bird brightness important in female choice which our scorers failed to pick up. Finally, as we indicate below, the scoring may depend on the quality of the illustrations used; at least two field guides were used in the original scoring (M. Zuk, personal communication), although we do not know which guide was used for which species.

These data suggest the need for independent scoring of bird brightness. We have therefore repeated the scoring procedure for European birds, because the scores used by A.F.R.² were those of Baker and Parker⁶, who had their own view concerning the evolution of bright colours, rather than those of independent assessors. We used the same six scorers and the same procedure and criteria, this time using Peterson *et al.*⁷ as our field guide.

We again take the brightness score for each species to be the average of the six new scores. Nine species are common to the European and North American data sets, so we compared the average brightness score for each of the nine species in the two regions to see whether there were differences which might be attributed, at least in part, to the use of different field guides. In eight of nine cases higher scores were given to the birds in the North American field guide (paired t -test, $t_{[8]} = 3.11$, P = 0.014; mean difference = 0.89). This suggests that our scores for the two regions are not comparable and we therefore treat the North American and European data separately.

Across European species, there is a significant positive correlation between parasite prevalence and male brightness (r_s = 0.29, n = 113, P = 0.002). Furthermore, a positive correlation is found within more taxa than expected by chance (33 of 47, P = 0.008). However, the correlation between parasite prevalence and brightness changes with the number of individual birds examined for parasites (n^*), which varies from one to 1,132 in the European data set: the correlation coefficient drops if species with small n^* are excluded. For example, if those species for which $n^* \leq 10$ are excluded from the analysis, the correlation coefficient between brightness and parasites across the remaining species almost halves and is not significant (r_s = 0.15, n = 78, P = 0.18). Conversely, if only species for which $n^* \leq 10$ are included, the association is highly significant (r_s = 0.51, n = 35, P = 0.003). This pattern is replicated within taxa. Among species for which $n^* > 10$, about half of the within-taxon associations are positive (19 of 39) whereas, for the remaining species ($n^* \leq 10$), 15 of 17 associations are positive (P = 0.002). Similar patterns are also found in the North American data set, but these are not replicated within taxa, so could be due to phylogenetic associations, although we can see no single taxon which might be responsible. Thus, within the European data set, and also possibly within the North American one, the strength of the association between brightness and parasites depends on the number of individuals sampled per species. The association, surprisingly, is strongest when relatively few individuals have been sampled.

None of the behavioural or ecological correlates of colour used by A.F.R.² varies with sample size in a way that would produce this effect. Nor are there any taxa that are sampled more often at low or high sample sizes which can account for the sample size effect. We are unable to explain this interaction with sample size, although we suspect that the solution lies in determining the factors (both biological and sociological) affecting parasite prevalence and n^* .

Therefore, the new brightness scores lead to different conclusions from those based on Hamilton and Zuk's scores for North American passerines^{1,2}, and the weak correlation we do find is associated with phylogeny. There is evidence for the predicted association between male brightness and haematozoan prevalence across European passerines when brightness is scored by independent assessors, but the relationship exists only across species in which few individuals have been sampled. Our analyses therefore do not provide general support for Hamilton and Zuk's prediction. Failure to find supporting comparative evidence does not necessarily mean that the hypothesis is wrong: there are several other possibilities^{2,8}, of which poor-quality data is probably the most obvious. Perhaps an accumulation of results similar to those reported here would allow acceptance of the null hypothesis of no association. Reanalysis of previous comparative tests reveal no significant relationships between parasites and sexually selected traits (reviewed in refs 8, 9). Apart from the relationships we have reported here, the only comparative survey that reports supporting evidence is Zuk's¹⁰ study of more than 500 species of Neotropical birds. □

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A response will be submitted by Marlene Zuk, to be published with a reply from A.F.R. and P.H.H. in a later issue of *Nature*.

Vesicular removal by oligodendrocytes of membrane attack complexes formed by activated complement

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OLIGODENDROCYTES synthesize myelin in the central nervous system and maintain it in lamellar sheaths around axons. Techniques for studying oligodendrocyte development *in vitro*¹ can be used, indirectly, to investigate the myelin injury that occurs in human and experimental demyelinating disease². Cell-mediated immune mechanisms are necessary but not sufficient to induce myelin damage *in vivo*; more recently complement has also been implicated in the pathogenesis both of multiple sclerosis^{3–5} and experimental allergic encephalomyelitis⁶. Previously we have demonstrated that antibody-independent complement activation occurs *in vitro* at the oligodendrocyte surface⁷. Here we show that the ensuing oligodendrocyte injury is reversible, and that recovery involves the release of membrane-attack complex-enriched vesicles from the surface of viable cells. The demonstration of morphologically and immunochemically identical vesicles in the cerebrospinal fluid of patients with multiple sclerosis suggests that reversible complement-mediated injury contributes to myelin damage *in vivo*.

Our preliminary studies extended reports of complement activation by purified myelin^{8,9}, and showed that exposure of intact neonatal rat optic nerve oligodendrocytes to homologous serum resulted in classical pathway complement activation in the absence of antimyelin antibodies⁷. Bipotential oligodendrocyte-type 2 astrocyte progenitor cells became increasingly susceptible to complement attack during differentiation and development into mature oligodendrocytes, whereas type 2 astrocytes did not activate complement.

During complement activation, terminal complexes form which insert into the cell membrane and act initially as calcium ionophores, later causing disruption of the cell¹⁰. We therefore measured (indirectly) the levels of intracellular free calcium in oligodendrocytes by using the calcium-sensitive photoprotein obelin¹¹ sealed within cells using temperature-sensitive liposomes¹² (Fig. 1). With maximum sublethal concentrations of

fresh rat serum alone (1:40 dilution), and in the presence of antibodies directed against the cell surface components galactocerebroside or myelin oligodendrocyte glycoprotein (serum at 1:60), oligodendrocytes were seen to recover from complement mediated attack through vesicular shedding of membrane-attack complexes. There was a cumulative effect of repeated exposure to individually sublethal levels of complement attack, implying that oligodendrocytes had a limited capacity for recovery.

The sequence of events leading to recovery involved an initial rise in intracellular free calcium, mainly derived from extracellular sources, although a significant release from intracellular stores was observed within 1–2 min of complement activation (Fig. 1). Intracellular calcium returned to normal within 5 min and was associated with a transient fall in ATP (data not shown). Under these conditions the cells remained impermeable to

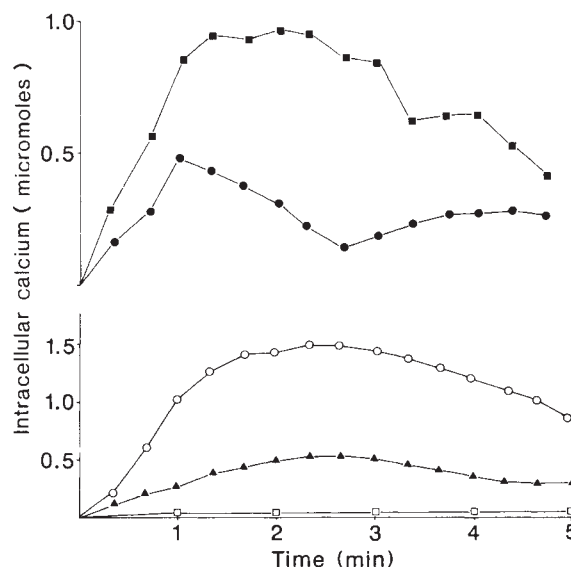


FIG. 1 Serial measurements of intracellular free calcium in oligodendrocytes preloaded with obelin and exposed to a sublytic concentration of complement alone (rat serum diluted 1:40) (●) or in the presence of anti-galactocerebroside antibody (10 $\mu\text{g ml}^{-1}$) and complement (rat serum diluted 1:60) (■). Intracellular calcium was also measured in cells exposed to complement (rat serum diluted 1:60) and anti-myelin oligodendrocyte glycoprotein antibody¹⁶ (3 $\mu\text{g ml}^{-1}$) in medium containing 1.3 mM calcium (○), in calcium-free medium containing 0.5 mM EGTA (▲) and after depletion both of extracellular and intracellular calcium using EGTA (□). Each point represents the mean from 4 separate experiments.

METHODS. Heat-sensitive liposomes were prepared by drying dipalmitoyl phosphatidylcholine in chloroform/methanol (3:1, v/v) under nitrogen to obtain a film of lipid which was resuspended in phosphate-buffered saline with 0.5 mM or 10 mM EGTA buffer (pH 7.4) containing obelin, isolated and prepared as previously described^{11,12}. Liposomes were fused with oligodendrocytes on coverslips by equilibration for 30 min at 4 °C in Krebs/HEPES buffer, pH 7.4, containing EGTA (0.5 mM) and D-glucose (16 mM). The coverslips were then replaced in the wells of a tissue culture plate and incubated in medium at 4 °C for 1 h. Finally, coverslips were washed carefully with medium before being incubated at 18 °C for 30 min, at 37 °C for 60 min and at 41 °C for 2 min to allow fusion of the liposomes with oligodendrocytes. Cells were then incubated with antibody for 20 min, washed with medium and replaced in wells. In experiments involving exposure to antibody and complement, oligodendrocytes were placed on a thermostatically controlled XY table and incubated with serum depleted of C9 (by passing normal serum over a column of Sepharose-anti-C9 antibody⁷) for 10 min to form C5b-8 complexes on the cell surface. C9 was added at time zero to form the membrane-attack complexes of complement and the coverslips were positioned in front of a photomultiplier tube connected to a locally constructed computerized chemiluminometer. Chemiluminescence counts were converted into measurements of intracellular free calcium as previously described¹⁷. The difference in magnitude between the calcium transients induced in the presence or absence of antibody may be partly dose-related, as different batches of rat serum varied slightly in cytotoxic potency. Liposome fusion with oligodendrocytes did not alter the threshold for complement-mediated injury.

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propidium iodide and the ability of immature oligodendrocytes to mature and express markers of more mature cells was not prejudiced. Serum depleted of C9 had no effect on membrane permeability or intracellular calcium, indicating that these changes depended upon the formation of membrane-attack complexes and not other biologically active products of complement activation such as C3a and C5a.

Oligodendrocytes were studied by electron microscopy to investigate this recovery mechanism (Fig. 2). Two minutes after exposure to maximum sublethal complement attack, the oligodendrocyte surface showed multiple vesicular extrusions which contained a high concentration of membrane-attack complexes identified with biotinylated anti-C9 antibody and gold-

labelled avidin. Within 6 min, oligodendrocytes resumed their pre-exposure appearances, having removed the vesicles. Vesiculation differed from patching, capping and shedding¹³ in that it occurred uniformly over the cell body and in the absence of antibody.

Particulate material, reactive with anti-C9 and galactocerebroside antibodies but not anti-myelin basic protein, was released into culture supernatants following sublethal complement attack, increasing in quantity between 2–5 min until reaching a plateau (Fig. 3a). This was not detected when oligodendrocytes were exposed to C9-depleted serum. The failure to bind antibodies to myelin basic protein, a component of oligodendrocyte and myelin membranes orientated exclusively at the cytosolic

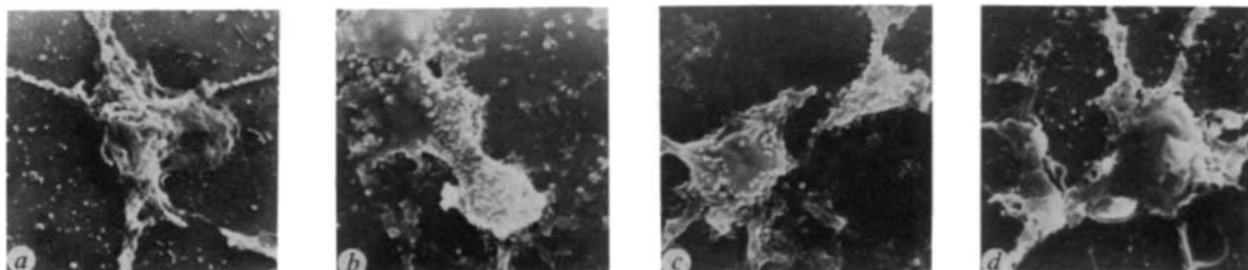


FIG. 2 Oligodendrocytes are shown before (a) and 3 min after (b, c) initiating non-lethal injury with complement. Numerous vesicular structures brightly stained with biotinylated anti-C9 antibody and avidin gold are seen on the cell body and its processes. Panel d shows an oligodendrocyte 6 min after initiating complement attack. Vesicles are no longer seen, although the oligodendrocyte appears swollen by comparison with the cell depicted in panel a.

METHODS. Optic nerves from 7-day-old Wistar rats were dissociated and cultured on poly L-lysine coated coverslips in Dulbecco's modified Eagle's

medium with Bottenstein-Sato additives, 0.5% heat-inactivated fetal calf serum and gentamicin ($25 \mu\text{g ml}^{-1}$) (ref. 1). After 5 days *in vitro*, cultured oligodendrocytes were exposed for 1–2 min to fresh rat serum (1:40 v/v), washed by dipping gently in fresh culture medium, then fixed with 2% paraformaldehyde at 0°C . Fixed cells were incubated with biotinylated anti-rat C9 antibody ($10 \mu\text{g ml}^{-1}$) for 15 min followed by gold-labelled avidin (Serotech, $10 \mu\text{g ml}^{-1}$) for 15 min and then subjected to dehydration, critical point drying and sputter coating with palladium-gold prior to imaging in a JEOL JSM 840 A scanning electron microscope at 18 kV.

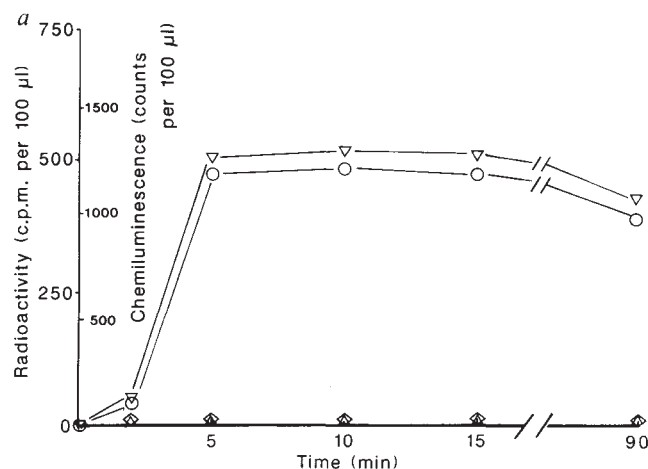
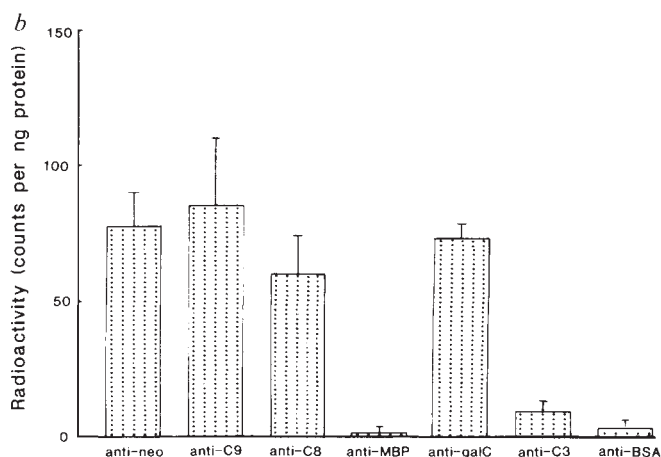


FIG. 3 Immunochemical characterization of vesicles found (a) in oligodendrocyte supernatants at various intervals during sublethal complement attack, and (b) in cerebrospinal fluid samples from patients with multiple sclerosis. a, The quantities of particulate material reactive with anti-rat C9 (∇), anti-myelin basic protein (Δ), anti-bovine serum albumin ($\circ$) and anti-galactocerebroside ($\circ$) antibodies are shown.

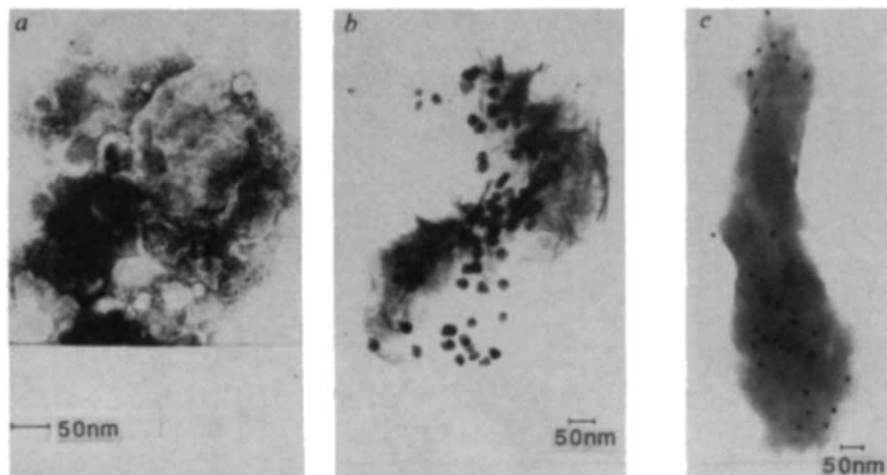
METHODS. a, Oligodendrocytes ($\sim 10^4$ per well) were sensitized with anti-galactocerebroside antibody and incubated with a sublytic dose (1:60) of homologous serum. Supernatant fluid (0.5 ml) was removed at intervals from individual wells, centrifuged to remove cell debris and divided into two portions. One was incubated with ^{125}I -labelled anti-rat C9 (10^6 c.p.m.) and with luminescence-labelled anti-human myelin basic protein (shown to cross-react with rat myelin basic protein by immunoblotting by B.P.M.) (10^6 counts); the other was incubated with ^{125}I -labelled anti-bovine albumin (10^6 c.p.m.) and luminescence labelled anti-galactocerebroside (10^6 counts) at 4°C for



15 min. Particle-bound antibody was separated on a column of Sepharose 2B and bound radio- and luminescence label quantified in the void volume peak at each time point using the mean of duplicate determinations. b, Portions of the Sepharose 2B void volume particulate material in cerebrospinal fluid samples from patients with multiple sclerosis were incubated with ^{125}I -labelled mono- or polyclonal antibodies to membrane-attack complex neoantigens, C9, C8, C3, (all prepared by B.P.M.), human myelin basic protein (a gift from Prof. R. Thompson), galactocerebroside and bovine serum albumin. About 2×10^6 c.p.m. of each labelled antibody were added to individual portions of the void volume peak. After 15 min at 4°C particle-bound and unbound antibody was separated and quantified as described above. Results are expressed as antibody counts bound per ng total protein in the particulate peak and are the means (with standard deviation) for samples of cerebrospinal fluid obtained from 3 individuals with multiple sclerosis.

FIG. 4 *a*, Ring lesions with the typical appearance of membrane attack complexes of complement are shown on vesicles recovered from the cerebrospinal fluid of patients with multiple sclerosis; panels *b* and *c* respectively illustrate surface binding of gold-labelled anti-membrane attack complex antibody and anti-galactocerebroside antibody on these vesicles.

METHODS. Aliquots of particulate material present in cerebrospinal fluid obtained from patients with multiple sclerosis and isolated by gel filtration on Sepharose 2B (relative molecular mass exclusion limit 4×10^7) were quantitated by absorbance at 280 nm, and placed directly on bacitracin-coated carbon collodion specimen grids for 1 h. Some grids were stained with biotinylated anti-rat C9 or anti-galactocerebroside antibodies and the appropriate gold-labelled second antibody. They were then washed and stained with 1% uranyl acetate prior to imaging at 80 kV in a Phillips 4000 transmission electron microscope.



or internal surface, suggests that the particulate material comprised intact vesicles rather than oligodendrocyte or myelin fragments.

The relevance of these *in vitro* observations was assessed by searching for vesicles in cerebrospinal fluid from patients with clinically definite or laboratory-supported probable multiple sclerosis¹⁴. Particulate material, reactive with antibodies to membrane-attack complex neoantigen, C9, C8, and galactocerebroside, but not with antibodies to myelin basic protein, bovine serum albumin or C3, was present in samples from 11 out of 19 patients with demyelination, irrespective of clinical disease activity (Fig. 3*b*), but not in any of 13 individuals with mechanical nerve root compression or 6 patients with intracranial mass lesions. Passage of labelled antibody-tagged particles through a 0.2 μ m filter removed over 75% of the radioactivity. Immune electron microscopy of the particulate material showed the presence of vesicles (0.1–0.5 μ m in diameter) bearing both membrane attack complexes and galactocerebroside (Fig. 4). It is difficult to be certain whether these vesicles originate from oligodendrocyte cell bodies or their myelin membrane extensions, or both, but discrete rounded lamellar myelin 'figures' of identical size to these vesicles have previously been detected in the cerebrospinal fluid of patients with multiple sclerosis¹⁵.

Recovery from complement-mediated cell injury by vesicular shedding of membrane-attack complexes occurs in a variety of human cell types¹⁰. The demonstration of this phenomenon in rat oligodendrocytes and the identification of vesicles in cerebrospinal fluid from patients with demyelination, but not in samples from normal controls or patients with structural CNS damage, has important implications for understanding the nature of multiple sclerosis. Our observations are consistent with the hypothesis that the immunopathogenesis of multiple sclerosis may ultimately involve complement-dependent injury of oligodendrocytes and their myelin membranes. Extensive zones where reversible injury has caused transient functional disturbance of myelinated axons may coexist with areas of oligodendrocyte depletion and demyelination resulting from intense or repeated complement attack. □

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Tolerance of class I histocompatibility antigens expressed extrathymically

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ALTHOUGH convincing evidence has been obtained for the imposition of self-tolerance by the intrathymic deletion of self-reactive T cells^{1–4}, the development of tolerance to antigens which are expressed only in the periphery is not so well understood. We have approached this question by creating transgenic mice⁵ which carry a class I major histocompatibility complex (MHC) gene (H-2K^b) linked to the rat insulin promoter⁶. Mice expressing the transgene develop diabetes, but do not appear to mount an immune response against the transgene-expressing pancreatic β -cells, even when the transgene is allogeneic with respect to the endogenous host H-2 antigens⁶. We have now explored the mechanism of this tolerance further. We find that spleen cells from pre-diabetic transgenic (RIP-K^b) mice do not kill targets bearing H-2K^b, whereas thymus cells from the same mice do. The unresponsiveness of these spleen cells can be reversed *in vitro* by providing recombinant interleukin-2 (rIL-2). In older, diabetic mice, responsiveness develops as the pancreatic β -cells are lost. Our results point to an extrathymic mechanism of tolerance induction, dependent on the continuous presence of antigen and the lack of IL-2 in the local environment of potentially reactive T cells.

In the RIP-K^b transgenic mice that expressed both membrane and cytoplasmic class I MHC molecules in their pancreatic β cells, diabetes developed early whether or not the mice were syngeneic with respect to the transgene product⁶. Transgene expression was detected in the pancreas⁶ but not in the thymus by immunoperoxidase staining (Fig. 1), northern blots or S1 nuclease mapping (not shown). Despite lack of thymic

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expression, lymphocytes were apparently not involved in the disease process as no detectable infiltration was ever observed in numerous histological examinations of the pancreas from mice of various ages and strains. Even when transgenic H-2^b mice expressing the H-2K^b molecule in their pancreatic β -cells were immunized with C57BL/6 (H-2^b) spleen cells, no cellular infiltrate was detected in the pancreata (not shown).

To test further the immunological status of RIP-K^b mice, spleen cells from 12-day-old transgenic mice were stimulated in a mixed lymphocyte reaction with B10.A(5R) cells (H-2D^d). Cytotoxicity tests were then performed on ⁵¹Cr-labelled H-2^b targets or, as an internal positive control, on H-2^d targets. Whereas spleen cells from either normal SJL mice or cells from nontransgenic littermates killed both targets, cells from the transgenic mice lysed the H-2^d targets but not the H-2^b targets (Fig. 2a, c). In contrast, transgenic spleen cells from older mice were able to kill both targets (Fig. 2b, d). These mice had advanced diabetes, with islets depleted of β -cells as judged by staining for insulin⁶. Thus, unresponsiveness was dependent upon the continued presence of tolerogenic amounts of the H-2K^b antigen.

If unresponsiveness was imposed in the periphery, rather than in the thymus, thymocytes should respond to H-2K^b-bearing cells. This was indeed the case (Fig. 3). Whereas splenocytes

were unresponsive to H-2K^b, the killing of H-2^b targets by thymus cells from the transgenic mice was not significantly different from that effected by cells from nontransgenic mice (Fig. 3). Thymus cells from both transgenic and nontransgenic mice also killed H-2^d targets (not shown). Tolerance was thus imposed upon H-2^b-reactive T cells at a stage after leaving the thymus, perhaps resulting from either the elimination of reactive cells following encounter with antigen or some reversible form of unresponsiveness⁷.

To distinguish between these possibilities, spleen cells from 14-day-old RIP-K^b transgenic mice were stimulated in the presence of added lymphokines. In preliminary experiments, coculture with supernatants from concanavalin A-stimulated splenocytes rendered the lymphocytes from transgenic mice as responsive to H-2K^b as those from nontransgenic littermates. Transgenic cytolytic T lymphocytes (CTL) generated without exogenous lymphokines had a specific defect in their ability to lyse H-2K^b targets (Fig. 4). Evidently, the concentration of IL-2 produced in mixed lymphocyte reactions (~ 1 U ml⁻¹; ref. 8) was not sufficient to reverse the nonresponsiveness of the H-2K^b-specific CTL. Whereas rIL-1 α improved the response only marginally, rIL-2 (at 10 U ml⁻¹) substituted for concanavalin A-stimulated splenocytes in inducing responsiveness. Tolerance is therefore due neither to clonal elimination nor permanent clonal

FIG. 1 Immunohistochemical staining of H-2K^b protein. Thymuses from 30-day-old transgenic RIP-K^b mice were analysed for H-2K^b using a two-stage immunoperoxidase assay. Thymus from a C57BL/6 mouse was used as a positive control for H-2K^b expression. a, C57BL/6 thymus. Positive staining is seen in medulla (M) and cortex (C). b, RIP-K^b thymus. No staining is detected. Nontransgenic littermates gave identical results. The scattered compact black staining in both a and b represents endogenous peroxidase from macrophages.

METHODS. Thymuses from the 31-2 lineage of the RIP-K^b transgenic mice backcrossed to SJL were processed for immunohistochemistry as described previously⁶, with the following modification. To decrease nonspecific background staining, the H-2K^b-specific monoclonal antibody, B8-24-3, was biotinylated and visualized using the avidin-biotin peroxidase coupled complex (Vectastain ABC kit). Tissues were also stained with a negative control monoclonal antibody specific for H-2K^k (11.4.1). No staining was observed with this antibody (not shown).

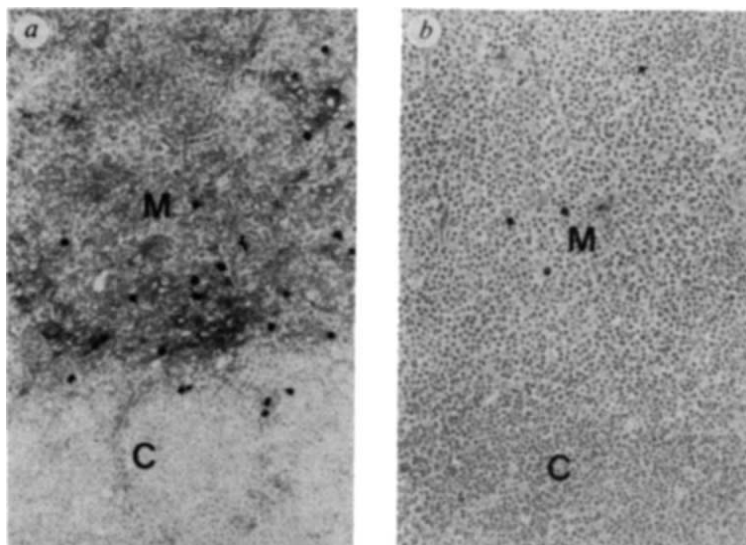
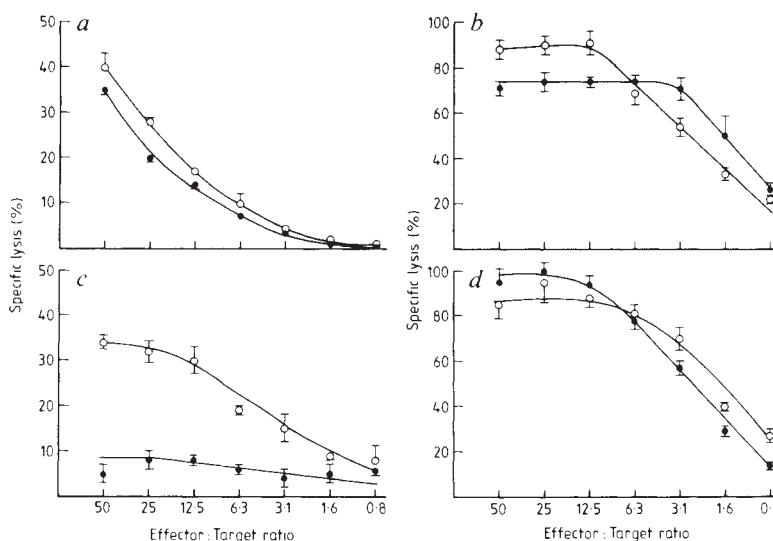


FIG. 2 Responsiveness of spleen cells to H-2K^b. Spleens were taken from RIP-H-2K^b mice (●) and their nontransgenic littermates (○) at 12 days (a, c) or 17 weeks (b, d). The cells were used to generate cytolytic T lymphocytes (CTL) as described below. The CTL were tested on P815 (a, b) and EL4 (c, d) targets.

METHODS. The spleen cells were cultured in 2 ml wells at 2×10^6 ml⁻¹ with an equal number of B10.A(5R) (H-2K^bD^d) irradiated (2,000 rads) stimulator cells. The culture medium was RPMI-1640 adjusted to mouse tonicity and supplemented with 10% fetal calf serum (Flow Laboratories) and 2-mercaptoethanol. After 4 days, cultures were harvested. CTL were counted and diluted twofold from a final effector:target (E:T) ratio of 50:1. Each dilution was tested in triplicate. ⁵¹Cr-labelled target cells, P815 (H-2^b) (a, b) or EL4 (H-2^b) (c, d), were added and the plates incubated for 4.5 h at 37 °C. The radioactivity released into 100 μ l supernatant was counted in a gamma counter (Packard, IL). Background release was measured from wells receiving target cells alone; maximum release was calculated from wells receiving 10 μ l of 10% Tween-20 (Sigma). Per cent specific lysis was calculated from the formula [(test release - background release)/(maximum release - background release)] $\times$ 100. Standard errors of the mean of triplicate samples are shown. Other experiments gave similar results. Although loss of tolerance in mice as young as 5 weeks was



observed in later experiments, older mice were used in this experiment to ensure maximum loss of β -cells.

energy, but to a specific defect in the supply of IL-2 to relevant lymphocytes.

Our experiments show that tolerance of class I-restricted T cells to extra-thymic H-2K^b is evident in the periphery, is reversible, and is dependent upon the continuous presence of a critical amount of antigen and on the lack of IL-2 in the local environment. IL-2 has been shown, previously, to abrogate the nonresponsiveness induced by incubating cloned T-cell lines with antigen in the absence of accessory cells *in vitro*⁹. Our system differs from this, however, in that tolerance was imposed to self-antigens *in vivo*.

Our results also differ from those of Lo *et al.*, who showed nonresponsiveness in thymocytes to class II molecules expressed by pancreatic β -cells¹⁰ (whether tolerance could be reversed was not determined). It was suggested that T cells from their transgenic mice had been inactivated as a result of aberrant antigen presentation by the class II-expressing pancreatic¹¹ β -cells, yet it is unclear how tolerance could have been achieved among thymocytes in these mice. Comparison of these observations with ours suggests the existence of distinct mechanisms responsible for inducing tolerance to extra-thymic antigens in class I and class II-restricted T cells. The difference may result from the separate processing pathways operating for the two classes of MHC molecules¹². Thus, extra-thymic antigens may be shed, processed and presented in association with class II molecules by cells which impose negative selection in the thymus. By contrast, antigens presented in association with class I molecules are usually synthesized intracellularly. This may account for the inability of soluble¹³ or extra-thymic class I molecules to impose tolerance by clonal elimination.

Taken together with the results of others, we suggest that tolerance is two-tiered. Cells which encounter antigens in the thymus are eliminated¹⁻³; these may include class-II-restricted T cells which see shed extra-thymic antigens presented intrathymically. By contrast, class-I-restricted CTL reactive to extra-thymic antigens are neither eliminated in the thymus nor permanently silenced. Indeed, there may be no need to impose

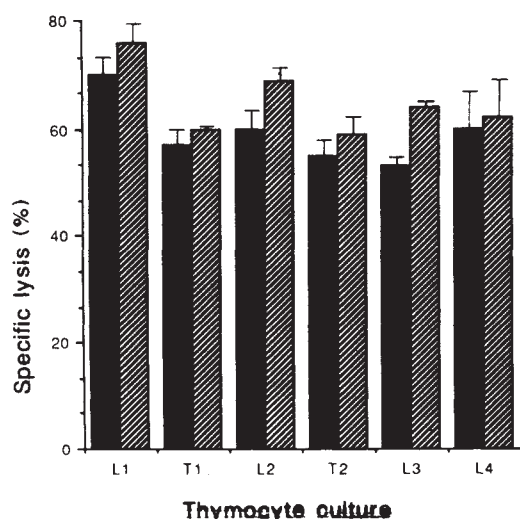


FIG. 3. Responsiveness of thymus cells to H-2K^b. The thymuses from 16-day-old mice of one litter were collected and cultured individually to generate CTL. The cells obtained from individual thymocyte cultures were analysed for their ability to lyse [⁵¹Cr] labelled EL4 targets.

METHODS. The cells were cultured at $5 \times 10^6 \text{ ml}^{-1}$ in the absence (black bars) or presence (striped bars) of added concanavalin A-stimulated splenocytes (2% v/v). CTL were generated as described in Fig. 2. The transgene status was subsequently determined: L1-4 refer to thymocyte cultures from nontransgenic littermates and T1-2 from transgenic mice. Thymocyte cultures from other litters gave similar results. Standard errors of the mean of triplicate samples tested at an E:T of 25:1 are shown.

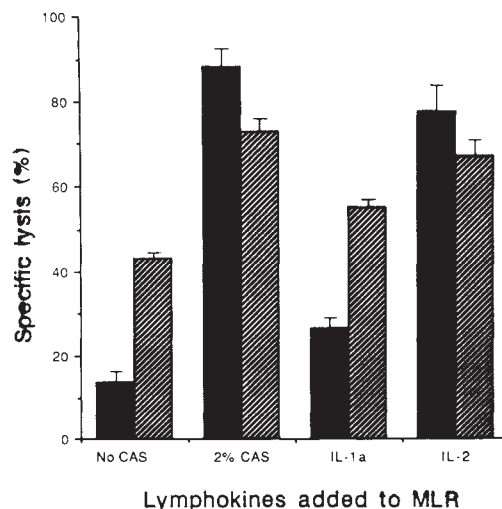


FIG. 4. Reversal of unresponsiveness by co-culture with IL-2. CTL were generated from spleen cells of transgenic mice and assayed for their ability to lyse EL4 (black bars) and P815 (striped bars) as described in Fig. 2, except that some cultures were supplemented with concanavalin A-stimulated splenocytes (2% v/v), rIL-1 (10 U ml^{-1}) or rIL-2 (10 U ml^{-1}). Lymphokines provided by Hoffmann-La-Roche and Cetus gave similar results. In the experiments shown here, the response of nontransgenic littermates to each target was also enhanced by co-culture with IL-2 (data not shown). Standard errors of the mean of triplicate samples tested at an E:T of 25:1 are shown.

tolerance on these cells *per se* if withholding 'help' ensures an effective state of tolerance *in vivo*, as observed here. Elimination or, alternatively, functional silencing of the 'helper' cells may be sufficient to maintain tolerance. For example, in the case studied here, the CD8⁺ helper cell subset for class I alloresponses¹⁴ could have been inactivated by inappropriate presentation in the periphery, as suggested for CD4⁺ cells in the class II β -cell transgenic experiments¹¹. Potentially reactive CTL would persist and be activated only when the concentration of tolerogen falls below some threshold (for example, in the older RIP-K^b mice, Fig. 2d) or upon delivery of both antigen and 'help' (for example, in the IL-2 supplemented CTL assays, Fig. 4).

Our results suggest a basis for the emergence of autoimmune diseases. Thus, tissue-specific class-I-restricted T cells may not be eliminated but instead remain dormant. If IL-2 is provided under abnormal conditions, a cascade of autoimmune reactions may be initiated. □

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Cyclosporin A inhibits activation-induced cell death in T-cell hybridomas and thymocytes

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ONE mechanism by which the immune system develops the ability to discriminate self from nonself is the deletion of autoreactive T-cell clones during thymic maturation¹⁻⁴. The drug cyclosporin A (CsA) has been shown to interfere with this process, allowing the escape of normally 'forbidden' T-cell clones^{5,6} and the appearance of autoimmune disease⁷⁻¹⁰. Recently, it has been demonstrated that immature thymocytes undergo programmed cell death (apoptosis) upon activation via the T-cell receptor¹¹. A similar phenomenon of activation-induced cell death (AICD) has been observed in T-cell hybridomas^{12,13}. Here we show that AICD in T-cell hybridomas *in vitro* and in thymocytes *in vivo* is blocked by CsA. Thus, clonal deletion may involve AICD when self-reactive, immature T cells are induced by self antigen, and CsA may cause autoimmunity by interfering with this process.

It has been suggested that AICD in thymocytes may be the mechanism by which autoreactive clones become negatively selected during T-cell development¹¹. As CsA has been shown to interfere with the process of negative selection in the thymus^{5,6}, we asked whether CsA blocks AICD. We first examined this in T-cell hybridomas. As shown in Fig. 1, loss of T-hybridoma cells was observed following activation by various stimuli. When CsA was added to cultures, cell death was inhibited (Figs 1 and 2). The effective doses of CsA corresponded to those required for inhibition of lymphokine production *in vitro*¹⁴. Similar results were obtained with another T-cell hybridoma, B1.1 (ref. 15) (data not shown).

Morphological changes associated with activation of T-hybridoma cells include chromatin condensation, membrane blebbing, and formation of small apoptotic bodies, all characteristics of apoptosis^{19,20}. The membrane changes can be readily observed at the light microscope level, as shown in Fig. 2, and could first be observed 5-7.5 hours after activation (not shown). CsA inhibited the AICD-associated formation of membrane blebs and apoptotic bodies.

During apoptosis, DNA within the affected cells undergoes fragmentation, revealed as a ladder of DNA fragments in multiples of 200 base pairs (bp) (refs 14, 15). As shown in Fig. 3, DNA from T-hybridoma cells undergoing AICD following activation with anti-CD3, showed this phenomenon. DNA from cells cultured with anti-CD3 in the presence of CsA, however, failed to show evidence of fragmentation. Thus, CsA prevents loss of cell viability, morphological changes and DNA fragmentation in T-hybridoma cells following activation via the T-cell receptor. This suggests that these phenomena are functionally related by a CsA-sensitive mechanism.

The observation that anti-CD3 antibodies can cause apoptosis in immature thymocytes in organ culture¹¹, suggested it might be possible to induce AICD in thymocytes *in vivo*. To test this idea, mice were injected, intraperitoneally (i.p.) with anti-CD3 antibody and thymocytes recovered 14 hours later. As shown in Fig. 4a, DNA from untreated thymocytes was intact, whereas the DNA in thymocytes taken from anti-CD3-treated animals was fragmented. Neither normal hamster serum nor monoclonal rat anti-L3T4 antibody (GK1.5) produced this effect. The DNA fragmentation was observed in thymus but not spleen or mesenteric lymph node. We then asked whether CsA, *in vivo*, could affect this phenomenon. Animals were injected with anti-CD3 (plus olive oil) or anti-CD3 plus CsA (in olive oil). The dose of CsA chosen was that demonstrated to interfere with clonal

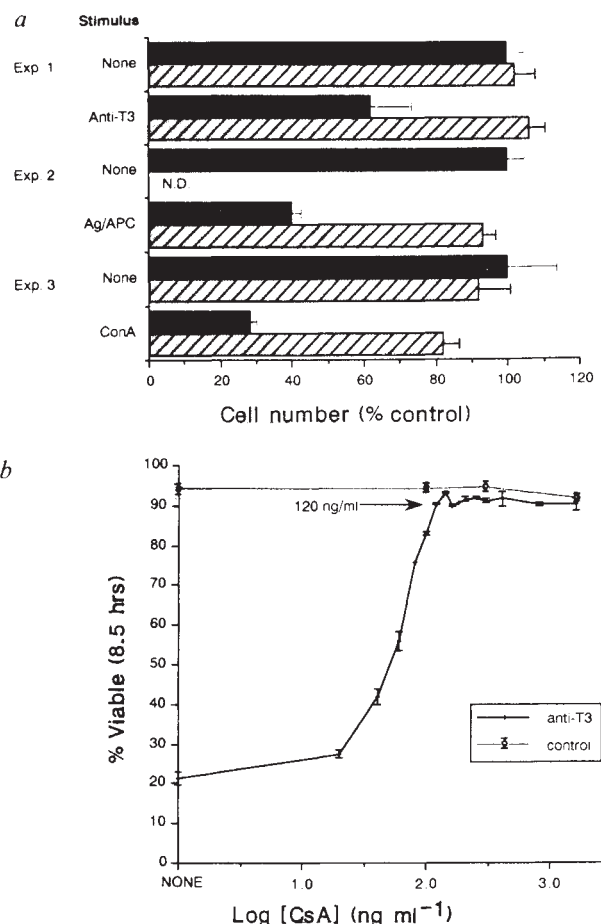


FIG. 1 Cyclosporin A blocks activation induced cell death in the T-cell hybridoma A1.1. **a**, Numbers of viable cells, as determined by MTT (tetrazolium) staining¹⁶, 24 h after activation of A1.1 cells with anti-T3 (ref. 17), specific antigen (poly 18, 20 µg per culture) plus antigen presenting TA3 cells (Ag/APC)¹⁵, or concanavalin A (conA) (5 µg per culture), with (cross-hatched bars) or without (black bars) CsA (200 ng ml⁻¹). Results represent mean % control cultures (without addition of stimulators or CsA) ± s.e. for three replicate wells. **b**, Titration of CsA added to cultures of A1.1 cells stimulated with anti-T3. Mean % viable cells ± s.e. of three replicate cultures was determined by exclusion of fluorescein isothiocyanate (FITC) in the presence of 5% fetal calf serum at 8.5 h post-stimulation. METHODS. Unless otherwise indicated, 96-well tissue culture plates (Costar) were incubated for 12 h with hamster anti-mouse T3 (114-2C11) in Tris-HCl, pH 9. Plates were washed and A1.1 cells added at 2×10^5 per well in 200 µl RPMI 1640 medium supplemented with 5% fetal calf serum. Viability was determined using MTT staining¹⁶ after 24 h (**a**) and viable cell number determined against standards. Alternatively, non-viable cells were identified by uptake of FITC (ref. 18) in the presence of 5% fetal calf serum and % viability determined by fluorescent microscopy (**b**).

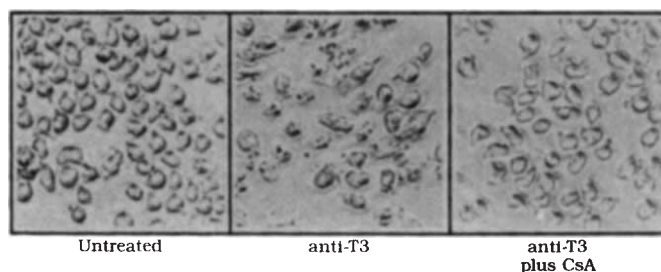


FIG. 2 Morphology of activated A1.1 cells. A1.1 cells were cultured in 96-well plastic tissue culture plates coated with hamster anti-murine T3 with or without 200 ng ml⁻¹ CsA (see legend to Fig. 1) and photographed using an inverted microscope after 12 h.

FIG. 3 CsA inhibits DNA fragmentation in activated A1.1 cells.

METHODS. A1.1 cells were cultured at 5×10^5 cells ml^{-1} in 10 ml RPMI 1640 plus 5% fetal calf serum in 75 cm^2 tissue culture flasks (Costar). Flasks were coated with anti-T3 as described in the legend to Fig. 1. CsA was added at 250 ng ml^{-1} . After 12 h, cells were harvested and washed at 4°C . Pellets were resuspended in 50 μl phosphate buffered saline followed by 2 ml digestion solution consisting of 0.5 M EDTA, 50 mM Tris-HCl (pH 8) containing 0.5% sodium lauryl sarkosinate and $100 \mu\text{g ml}^{-1}$ proteinase K. Digestion was carried out at 50°C for 3 h. The solution was extracted with an equal volume of equilibrated phenol, then phenol/chloroform, then equilibrated chloroform. The DNA solution was dialyzed against four changes of 6 litres 50 mM Tris-HCl, 10 mM EDTA, 10 mM NaCl (pH 8.0) at 4°C . RNase A ($200 \mu\text{g ml}^{-1}$) was added to each sample and incubated at 37°C for 3 h. DNA was then precipitated with 66% ethanol in the presence of 0.3 M sodium acetate, collected, and washed once with 80% ethanol. After drying, DNA was dissolved in 10 mM Tris-HCl, 1 mM EDTA (pH 8.0). Twenty μg of DNA were mixed with 0.25% bromophenol blue and 40% sucrose before loading into wells of a 2% agarose gel. Electrophoresis was carried out in 80 mM tris-phosphate, 2 mM EDTA (pH 8.0). After electrophoresis, gels were stained with $0.1 \mu\text{g ml}^{-1}$ ethidium bromide and visualized by ultraviolet light. Units in kilobases.

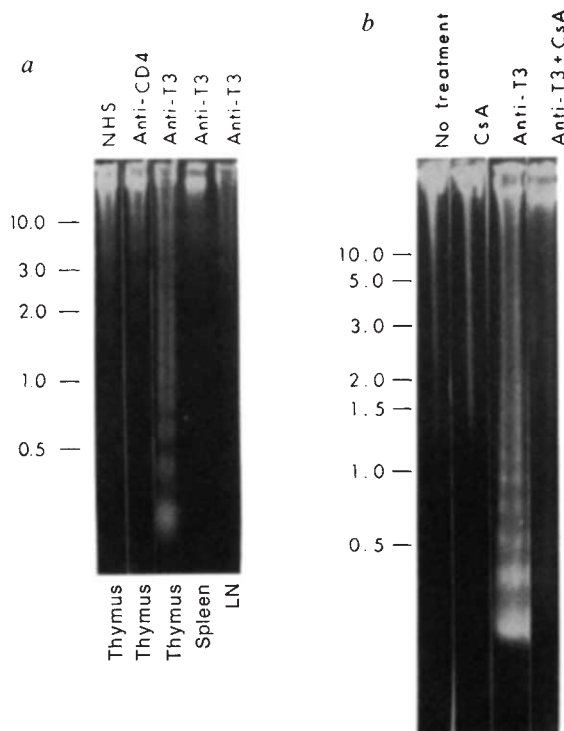
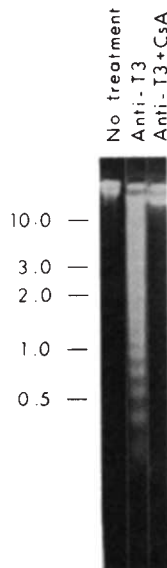


FIG. 4 CsA inhibits DNA fragmentation in thymocytes following intraperitoneal injection of anti-T3.

METHODS. a, Three week old Balb/cCr mice (University of Alberta Animal Breeding Facility) were injected with $200 \mu\text{l}$ i.p. NH_4SO_4 -precipitated normal hamster serum (NHS), rat anti-mouse L3T4 (GK1.5), or hamster anti-mouse T3 (114-2C11, ref. 17). Spleen, mesenteric lymph node (LN) and thymus were taken from each animal 18 h after injection and DNA extracted as in the legend to Fig. 3. Results shown are representative of 3 mice per group. No DNA fragmentation was observed in spleen or lymph node of NHS or anti-L3T4 injected animals (not shown). b, Three-week-old Balb/cCr mice were injected intraperitoneally with saline or anti-T3 as in (a) above. Animals also received 25 mg per kg bodyweight CsA in $200 \mu\text{l}$ olive oil (or olive oil alone). After 24 h, the thymus was removed and DNA extracted as above. The observed effect of CsA plus anti-T3, versus anti-T3 alone was representative of 6 out of 8 animals analysed 14–24 h after injection. Units in kilobases.

deletion in developing thymocytes⁵. CsA dramatically reduced the thymic DNA fragmentation after anti-CD3 treatment *in vivo* (Fig. 4b).

One suggested mechanism by which CsA might interfere with clonal deletion and acquisition of self tolerance *in vivo*, is by down-regulating the expression of class II major histocompatibility complex molecules on antigen-presenting cells in the thymus⁹. Our results suggest that an alternative mechanism of action of CsA is by direct interference with the intracellular deletional process. Clearly, an interesting possibility is that CsA interferes with AICD by blocking activation of a gene and/or biochemical pathway directly involved in programmed cell death, following activation via the T-cell receptor. In this respect, it is relevant that AICD in T-cell hybridomas can be blocked by actinomycin D or cycloheximide (Y.S. *et al.*, in preparation). Because the action of CsA is much more selective than are these drugs¹⁶, the effect of CsA on AICD both *in vitro* and *in vivo* may provide important clues to the mechanism of programmed cell death. □

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Dephosphorylation and activation of *Xenopus* p34^{cdc2} protein kinase during the cell cycle

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GENETIC studies in the fission yeast *Schizosaccharomyces pombe* have established that a critical element required for the G2→M-phase transition in the cell cycle is encoded by the *cdc2*⁺ gene^{1–5}. The product of this gene is a serine/threonine protein kinase, designated p34^{cdc2}, that is highly conserved functionally from yeast to man² and has a relative molecular mass of 34,000 (34 K). Purified maturation-promoting factor (MPF) is a complex of p34^{cdc2} and a 45K substrate that appears in late G2 phase and is sufficient to drive cells into mitosis^{6–9}. This factor has been iden-

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tified in all eukaryotic cells^{10,11}, and *in vitro* histone H1 is the preferred substrate for phosphorylation^{7,9,12}. The increase in the activity of H1 kinase in M-phase is associated with a large increase in total cell protein phosphorylation which is believed to be a consequence of MPF activation¹³⁻¹⁸. We show here that the H1 kinase activity of p34^{cdc2} oscillates during the cell cycle in *Xenopus*, and maximal activity correlates with the dephosphorylated state of p34^{cdc2}. Direct inactivation of MPF *in vitro* is accompanied by phosphorylation of p34^{cdc2} and reduction of its protein kinase activity.

To investigate whether p34^{cdc2} in *Xenopus* MPF is directly regulated by M-phase phosphorylation, we immunoprecipitated p34^{cdc2} with PSTAIR antibody⁶ from either G2-phase oocytes or M-phase oocytes at germinal vesicle breakdown (GVBD) after pulse-labelling by injection of [γ -³²P]ATP. As shown in Fig. 1, lanes 1 and 2, total oocyte protein phosphorylation was markedly elevated in M-phase, as reported previously¹⁷. But, the phosphorylation state of p34^{cdc2} was high in G2-phase oocytes and very low in GVBD oocytes (Fig. 1, lanes 3 and 4). This difference in ³²P_i-content of p34^{cdc2} is unlikely to represent changes in turnover, because prelabelling of p34^{cdc2} before progesterone induction of GVBD also demonstrated almost complete dephosphorylation during oocyte maturation (data not shown). To investigate any relationship between the phosphorylation and activity of p34^{cdc2}, time-course studies were carried out on progesterone-treated oocytes undergoing maturation. As shown in Fig. 2a and b, western blotting of total oocyte lysates with the PSTAIR antibody during maturation revealed no noticeable change in the amount of p34^{cdc2}, even though a 10-fold increase in the activity of H1 kinase occurred. Immune-complex kinase assays demonstrated a similar increase in PSTAIR immunoprecipitates, confirming that the increase in kinase activity in lysates can be accounted for at least in part

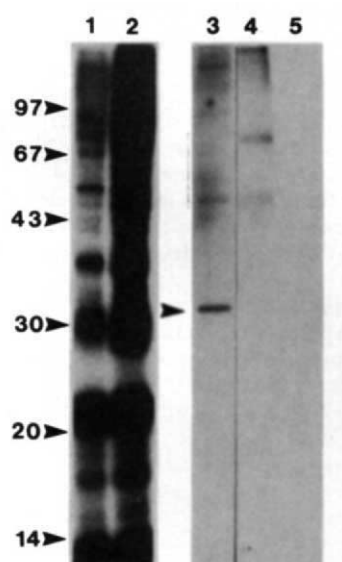


FIG. 1 Comparison of total phosphorylation with p34^{cdc2} phosphorylation in G2 and M phase. Oocytes arrested in G2 (lane 1) or just after progesterone-induced GVBD (lane 2) were microinjected with 10 μ Ci of [γ -³²P]ATP, labelled for 1 h, then lysed in 10 μ l per oocyte of buffer containing 80 mM β -glycerophosphate, 50 mM NaF, 20 mM EGTA, 15 mM MgCl₂, 20 mM HEPES (pH 7.5), 1 mM dithiothreitol, 0.3 mM phenylmethylsulphonylfluoride and leupeptin (0.3 μ g ml⁻¹), and centrifuged for 2 min at 15,000 g. An aliquot of the clear supernatant corresponding to half an oocyte was electrophoresed on 10% gels and autoradiographed. The same lysates were subjected to immunoprecipitation with the PSTAIR antibody as previously described⁶. Lane 3: prophase-arrested oocytes, lane 4: oocytes following GVBD. Lane 5 is a control in which prophase-arrested oocytes were subjected to immunoprecipitation with PSTAIR antibody preincubated for 1 h at 4°C with the PSTAIR peptide (1 ng per μ l of antibody). Molecular weight markers are shown on the left (in thousands).

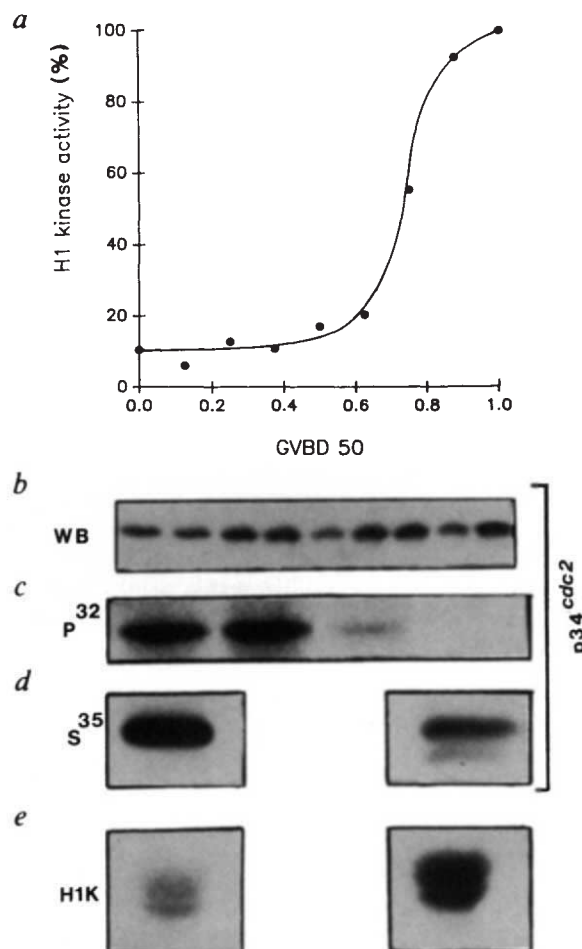


FIG. 2 Regulation of histone H1 kinase activity and p34^{cdc2} during progesterone-induced oocyte maturation. a, Histone H1 kinase activity during maturation. At various times after addition of progesterone, oocytes were lysed as described in Fig. 1 and assayed for histone H1 kinase activity as described previously⁶. The results are expressed as fractional periods of the time to 50% germinal vesicle breakdown. 1.0 GVBD50 occurred at 240 min post-progesterone. b, Western blot analysis of p34^{cdc2}. Equal amounts of protein from the same lysates as in a were electrophoresed on 12.5% acrylamide gels and western blotted with the PSTAIR antibody as described previously⁶. Although not evident in this figure, longer exposure of the blot revealed the presence of a faster-moving form. The nine lanes correspond (from left to right) to 0.0, 0.125, 0.25, 0.357, 0.5, 0.625, 0.75, 0.875, 1.0 GVBD50. c, p34^{cdc2} phosphorylation during maturation. At different times during progesterone-induced maturation, five oocytes were microinjected each with 10 μ Ci of [γ -³²P]ATP, labelled for 25 min, then lysed and immunoprecipitated as previously described (Fig. 1). The lanes are (from left to right): G2-arrested oocytes (non-hormone-treated), oocytes labelled from 0.2 to 0.3 GVBD50, oocytes labelled from 0.5 to 0.6 GVBD50, and oocytes labelled from 0.9 to 1.0 GVBD50. d, Synthesis of p34^{cdc2} during maturation. Oocytes labelled for 1 h after injection of [³⁵S]methionine were processed for immunoprecipitation with the PSTAIR antibody. The two lanes are: prophase-arrested oocytes (left) and oocytes labelled immediately following GVBD (right). Only the region of the gel corresponding to p34^{cdc2} is shown. e, Immune-complex histone H1 kinase activity from oocytes in G2 or following GVBD. After immunoprecipitation with the PSTAIR antibody the kinase activity was measured as described previously⁶. Only the region of the gel corresponding to histone H1 is shown.

METHODS. The H1 kinase assay was performed in a final volume of 30 μ l containing 20 mM HEPES, 30 mM β -mercaptoethanol, 0.1 mg ml⁻¹ bovine serum albumin, 10 mM MgCl₂, 0.5 mg ml⁻¹ purified calf thymus histone H1, 0.2 μ g heat-stable inhibitor of cAMP-dependent protein kinase and 0.1 mM ATP containing 2×10^7 c.p.m. of [γ -³²P]ATP. Reactions were incubated at 30°C for 15 min, stopped by addition of 1/4 volume of 5 $\times$ electrophoresis sample buffer and processed for SDS-PAGE. The radioactivity transferred to the histone H1 was then measured by scintillation counting of the excised gel band.

by $p34^{cdc2}$ (Fig. 1e). The kinetics of the increase in the activity of H1 kinase at 0.6 GVBD50 correlate with the appearance of MPF and the burst of phosphorylation observed in cells entering M-phase^{17,18}.

To study the phosphorylation state of $p34^{cdc2}$ during its activation, the PSTAIR antibody was used to immunoprecipitate $p34^{cdc2}$ from oocytes pulse-labelled by [³²P]ATP-injection at various times during maturation. As shown in Fig. 2c, before the increased activity of H1 kinase at 0.6 GVBD50, $p34^{cdc2}$ was highly phosphorylated, but it became dephosphorylated just before the increase in H1 kinase activity. Complete dephosphorylation was also evident in oocytes prelabelled with [³²P]phosphate before progesterone addition (data not shown). This result suggests that the H1 kinase activity of MPF is activated upon dephosphorylation of $p34^{cdc2}$. Further evidence that dephosphorylation occurred was obtained by analysing the synthesis of $p34^{cdc2}$ in maturing oocytes treated with progesterone and pulse-labelled by injection of [³⁵S]methionine. As shown in Fig. 2d, the protein was synthesized in approximately equal amounts in both the presence and absence of progesterone. Of interest however, was the finding that in oocytes that had entered M-phase and undergone GVBD, newly synthesized $p34^{cdc2}$ appeared as a doublet, reflecting the appearance of a faster

moving form with an apparent molecular weight of 32 K. The slower form of $p34^{cdc2}$ labelled with [³⁵S]methionine migrated with the same molecular weight as the phosphorylated form labelled with [³²P]ATP. The faster moving form could also be detected in western blots exposed longer than in Fig. 1b and accounted for about 10% of total $p34^{cdc2}$ synthesis at GVBD. The presence of the faster-migrating form is consistent with the dephosphorylation of $p34^{cdc2}$ evident in ³²P-labelling (Fig. 1c). The finding here that only a subfraction of $p34^{cdc2}$ molecules undergo dephosphorylation in M phase is consistent with results in other cells which indicate that only a small fraction of $p34^{cdc2}$ molecules participate in formation of high molecular complexes with other proteins and exhibit elevated protein kinase activity^{12,19}. In HeLa cells the $p34^{cdc2}$ incorporated into such a complex was reported to undergo increased phosphorylation, but possible dephosphorylation was also noted as cells progressed into late G2 (ref. 19).

MPF activity is known to be high in unfertilized eggs of various species and to oscillate with the cell cycle in the rapid divisions that follow fertilization or activation^{18,20,21}. These oscillations have in some cases been reported to occur in concert with changes in histone kinase activity. To determine whether this situation in *Xenopus* reflects $p34^{cdc2}$, eggs were parthenogenetically activated with the calcium ionophore A23187 and examined for changes in histone kinase activity during the first cell cycle. As shown in Fig. 3a, upon activation H1 kinase activity abruptly fell 10-fold and remained low until shortly before the first mitosis, when it returned to a level near that of the unfertilized egg. Shortly after completion of first mitosis but before cytokinesis, H1 kinase activity again decreased about 10-fold. The kinetics of these oscillations are similar to those of MPF activity^{20,21} and similar oscillations were seen when eggs were fertilized rather than activated (data not shown). This oscillation in H1 kinase activity was not accompanied by any important change in the total amount of $p34^{cdc2}$ itself (Fig. 3b). But upon injection of [³²P]ATP into eggs at the time of activation, it was evident that $p34^{cdc2}$ became phosphorylated when H1 kinase activity decreased after activation and dephosphorylated when H1 kinase activity increased again at first mitosis (Fig. 3c). Immune-complex kinase assays confirmed these oscillations in H1 kinase activity reflected changes in $p34^{cdc2}$ (Fig. 3d). These results support further the view that cell-cycle-dependent oscillations in H1 kinase activity are due to $p34^{cdc2}$ in MPF and that the activity of the kinase is decreased when it becomes phosphorylated.

These results demonstrate a strong inverse correlation between the *in vivo* phosphorylation state of $p34^{cdc2}$ and its protein kinase activity, suggesting that phosphorylation inactivates the enzyme.

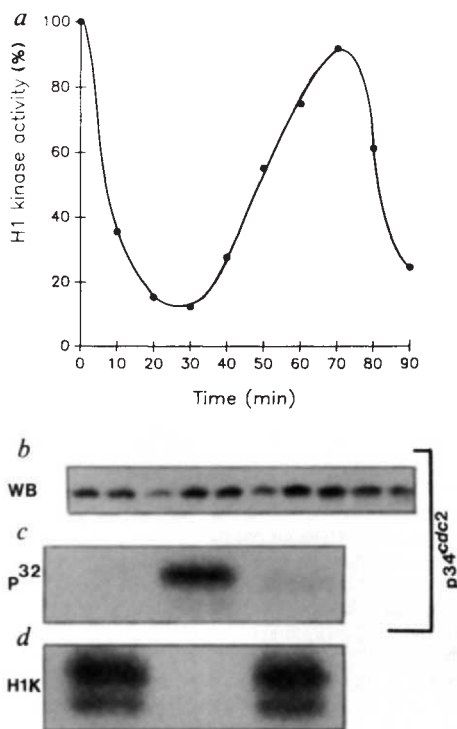


FIG. 3 Histone H1 kinase activity and regulation of $p34^{cdc2}$ after egg activation. **a**, Oscillation of histone H1-kinase activity in egg lysates. The assays were performed as for Fig. 2a. 0: time of *in vitro* activation by the calcium ionophore A23187. **b**, Western blot analysis showing the amount of $p34^{cdc2}$ during the first cell cycle. The ten lanes correspond (from left to right) to the following times: 0, 10, 30, 40, 50, 60, 70, 80, 90 and 100 min following activation. **c**, Levels of $p34^{cdc2}$ phosphorylation during the first cell cycle in activated eggs. The first lane represents unfertilized eggs microinjected and labelled for 30 min with [³²P]ATP. Mechanical activation due to the microinjection was prevented by incubating the eggs for 15 min before injection and throughout the labelling period in MBS containing 10 mM chlorobutanol. The second lane represents eggs mechanically activated during microinjection of [³²P]ATP and labelled for 30 min; the third lane is similar except labelling is for 70 min. **d**, Immune-complex histone H1 kinase activity from unfertilized eggs. The first lane represents unfertilized eggs, the second lane eggs 30 min after ionophore activation, and the third lane eggs 70 min after activation.

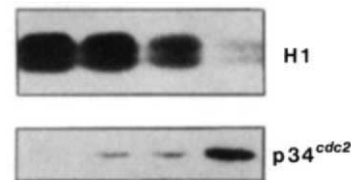


FIG. 4 *In vitro* phosphorylation and inactivation of $p34^{cdc2}$. Metaphase extracts were prepared by crushing unfertilized eggs in the presence of 10 mM EGTA as described previously²². 1×10^8 c.p.m. of [³²P]ATP was added to some samples and then various amounts of 50 mM calcium chloride were added to 20 μ l of the extracts to the following final calcium concentrations; lane 1, no Ca^{2+} ; lane 2, 0.2 mM Ca^{2+} ; lane 3, 0.5 mM Ca^{2+} ; lane 4, 2.0 mM Ca^{2+} . H1 kinase activity in the unlabelled extract and the [³²P]-phosphate content of $p34^{cdc2}$ in the labelled extract were measured as in Fig. 1. This procedure has been found to inactivate MPF activity and cause chromosomes to decondense and reform a nuclear envelope²². The data show direct *in vitro* inactivation of MPF is accompanied by a progressive reduction in H1 kinase activity and increasing phosphorylation of $p34^{cdc2}$.

To provide a more direct test of whether phosphorylation exerts a causal effect on the protein kinase activity, metaphase extracts containing active MPF were prepared from unfertilized eggs and incubated with [γ - 32 P]ATP. We have previously shown that addition of calcium to such preparations inactivates MPF and converts chromosomes into interphase nuclei²². As shown in Fig. 4, *in vitro* inactivation of MPF led to a marked decline in H1 kinase activity, and p34^{cdc2} became heavily phosphorylated. In attempts to perform the converse experiment, we have so far been unable to dephosphorylate interphase p34^{cdc2} with protein phosphatases 1 or 2A, or acid or alkaline phosphatase. Possibly only p34^{cdc2} complexed with other proteins at the G2/M border is susceptible to dephosphorylation. The direct phosphorylation and inactivation of p34^{cdc2} *in vitro* (Fig. 4), however, provides strong evidence for a causal effect of phosphorylation on p34^{cdc2}. The apparent inactivation of p34^{cdc2} by phosphorylation is only the second example of direct inactivation of a serine/threonine kinase by another kinase, the classic case being phosphorylation and inactivation of myosin light-chain kinase by cAMP-dependent protein kinase²³.

It has long been known from genetic studies in *S. pombe* that expression of the *cdc2*⁺ gene is essential for the G2→M transition⁵. Its activity appears to be highly regulated because of the large number of other *cdc* genes that control its function. Notable among these other elements are the products of the *wee1*⁺ gene, whose expression inhibits *cdc2*⁺ function, and the *nim1*⁺ gene, whose product activates *cdc2*⁺ by a mechanism that involves *wee1*⁺ (refs. 24, 25). Both *nim1*⁺ and *wee1*⁺ protein products contain consensus sequences for protein kinases. The possibility suggested by these genetic studies, that *wee1* might directly inactivate p34^{cdc2} by phosphorylation, is consistent with our biochemical finding that inactivation of p34^{cdc2} kinase activity in the cell cycle is correlated with an increase in its 32 P content (Figs. 2–4). But, further work is needed to identify directly the protein kinase(s) that phosphorylate p34^{cdc2} during the cell cycle.

The oscillations in H1 kinase activity presented here follow closely the reported oscillations in MPF activity during oocyte maturation and after egg activation^{20,21}. At present we do not know if the changes in phosphorylation of p34^{cdc2} and its kinase activity are sufficient to regulate MPF activity or whether additional changes in the association or activity of the 45K subunit of MPF are also required. Because p45 is a substrate for p34^{cdc2} in the MPF complex⁹, it is possible p45 phosphorylation would decrease upon p34^{cdc2} inactivation and be important for loss of MPF activity. In any case it seems likely that phosphorylation and inactivation of p34^{cdc2} contribute to regulation of MPF activity during the cell cycle. The convergence of evidence for this from both the biochemical and genetic point of view suggests further work on this regulation in the *Xenopus* egg cell-free system will lead to isolation of the kinase(s) that regular p34^{cdc2}. □

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Glucocorticoid- and progesterone-specific effects are determined by differential expression of the respective hormone receptors

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ALTHOUGH glucocorticoids and progestins control vastly different physiological processes, the receptors mediating the effects of these hormones interact with the same DNA sequences^{1–4}. Transfer experiments involving synthetic genes⁵ and *in vitro* binding studies⁶ have shown that progesterone and glucocorticoid receptors both recognize the same 15-base pair DNA element (TGTACAGGATGTTCT), raising the question of how the two steroids affect gene expression selectively. We considered the possibility that their selectivity arises from either the differential expression of the receptors in target cells or the differential dependence of receptor function on additional transcription factors. To test these alternatives we introduced a progesterone-receptor expression plasmid into the rat hepatoma cell line Fto2B-3 which contains glucocorticoid receptor but is devoid of progesterone receptor. We report that expression of the progesterone receptor in Fto2B-3 cells renders endogenous glucocorticoid-regulated genes inducible by progestins. Our data show that the responsiveness of a cell to external stimuli can be reprogrammed by the expression of a single transcription factor and that differential expression of hormone receptors is at least one mechanism by which steroid-specific gene activation is achieved.

To investigate whether glucocorticoid-regulated genes in the liver, a major target for glucocorticoids which does not express progesterone receptor⁷, could be made progesterone-responsive we stably introduced an expression plasmid coding for the chicken progesterone receptor⁸ (Fig. 1a) into Fto2B-3 rat hepatoma cells^{9,10}. In two of the stably transformed clones (clone 1, clone 2) the levels of progesterone receptor were measured by steroid-binding assays¹¹ (Fig. 1b). To assay for functional progesterone receptor we analysed induction mediated by the 15-base pair (bp) progestin/glucocorticoid response element (GRE) upstream of the thymidine kinase (*Tk*) promoter⁵ in transient transfections of clone 1 and clone 2 cells (Fig. 1c). Administration of either the synthetic progestin R5020 or the synthetic glucocorticoid dexamethasone resulted in similar inductions of chloramphenicol acetyltransferase (CAT) expression from the plasmid pGRE15A (ref. 5) in clone 1 and clone 2 cells. R5020 did not induce CAT expression in the parental cell line Fto2B-3, showing that the induction of *Tk*CAT expression by R5020 is due to specific activation of the chicken progesterone receptor in clone 1 and clone 2 cells.

To test whether expression of the progesterone receptor in clone 1 and clone 2 cells would also allow an endogenous, glucocorticoid-regulated gene to be induced by progestins,

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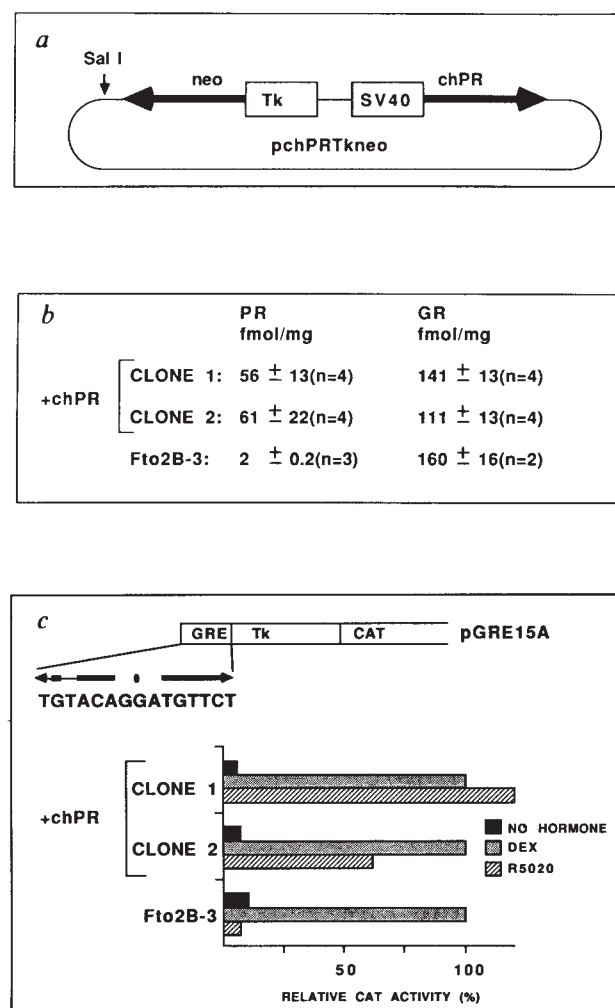


FIG. 1 Transformation of rat hepatoma cells with the chicken progesterone receptor. **a**, Map of the chicken progesterone-receptor expression plasmid pchPRTkneo, which contains the cDNA of the B-form of the chicken progesterone receptor (chPR) linked to the SV40 early promoter/enhancer region⁸ and the neomycin resistance gene (*neo*) under control of the thymidine kinase promoter of Herpes simplex virus (*Tk*). **b**, Determination of glucocorticoid receptor (GR) and progesterone receptor (PR) levels in cells of clone 1 and clone 2 (Fto2B-3 cells stably transfected with the plasmid pchPRTkneo) and the parental Fto2B-3 cells. Results are expressed as fmol of receptor per milligram of extract protein. Standard deviations were calculated from the results of several determinations (n =number of measurements). The low level of specific binding of R5020 in Fto2B-3 extracts is most likely a consequence of cross-reaction of R5020 with the glucocorticoid receptor¹⁹. **c**, CAT expression from plasmid pGRE15A is induced equally by R5020 and dexamethasone in cells of clone 1 and clone 2. The plasmid pGRE15A (ref. 5) contains a 15-bp progestin/glucocorticoid response element (GRE) upstream of the thymidine kinase gene promoter (*Tk*) of Herpes simplex virus which drives the expression of CAT. The structure of the plasmid pGRE15A and the sequence of the GRE are shown. pGRE15A was transiently transfected by electroporation. Clone 1, clone 2 and Fto2B-3 cells were induced by either 10 nM R5020, 100 nM dexamethasone (DEX) or no hormone. The results are expressed relative to the glucocorticoid-induced CAT activity which is set to 100%.

METHODS. Details of the construction of pchPRTkneo are available on request. Receptor concentration was measured by the charcoal assay¹¹. Clone 1 and clone 2 cells were kept in medium containing 800 $\mu\text{g}/\text{ml}^{-1}$ G418 and grown as previously described⁹. To generate transformants expressing the chicken progesterone receptor, Fto2B-3 cells were transfected with pchPRTkneo linearized at the *Sal*I site (see Fig. 1a) by either electroporation⁹ or by calcium phosphate coprecipitation²⁰. Single colonies were screened for progesterone receptor expression by analysing the effect of R5020 and dexamethasone on the expression of transiently transfected pMMTVCAT (ref. 21). Transient transfections, hormone treatment and determination of CAT enzymatic activity were performed as previously described⁹.

the effects exerted by R5020 on expression of the tyrosine aminotransferase gene¹² (TAT, Fig. 2a), the carbamoylphosphate synthetase I (CPS) gene and the argininosuccinate synthetase (ASS) gene (Fig. 2b) were analysed. The messenger RNA levels for all three genes were elevated in response to R5020 in transformants expressing chicken progesterone receptor but not in the parental Fto2B-3 cells. Glucocorticoid-regulated genes were, thus, rendered equally responsive to progestins simply by the presence of the progesterone receptor. Hence, receptor content is decisive for steroid selective activation of these genes.

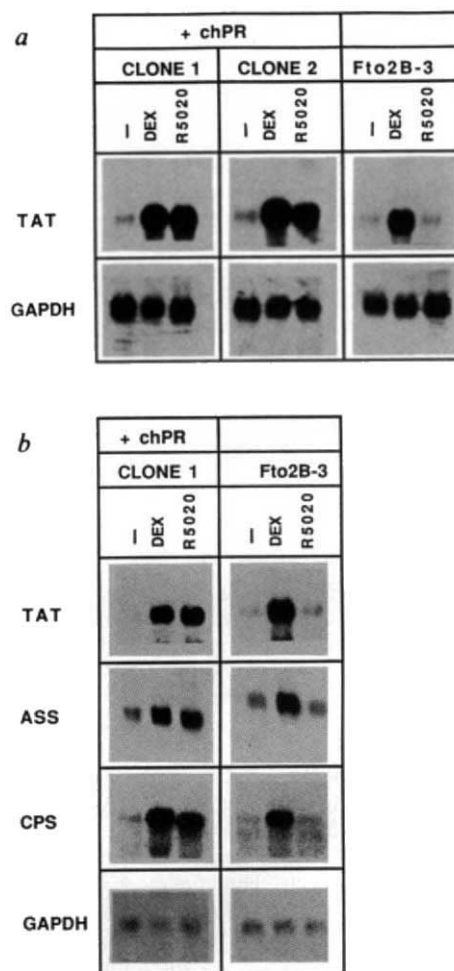


FIG. 2 Expression of the chicken progesterone receptor renders endogenous glucocorticoid-regulated genes inducible by the synthetic progestin R5020. **a**, Northern-blot analysis of tyrosine aminotransferase (TAT) mRNA in clone 1, clone 2 and Fto2B-3 cells following administration of dexamethasone (DEX) or R5020. **b**, Argininosuccinate synthetase (ASS) and carbamoylphosphate synthetase I (CPS) mRNA levels following R5020 or dexamethasone administration. Cells were treated with either no hormone (—), or 10 nM R5020 or 100 nM dexamethasone (DEX). Total RNA was isolated and analysed by the northern-blotting procedure using antisense cDNA probes specific for the various mRNAs. As a control (**a** and **b**) the same filters were rehybridized with a probe specific for glyceraldehyde 3-phosphate dehydrogenase mRNA (GAPDH).

METHODS. Cells were treated for 12 h with either 100 nM dexamethasone, 10 nM R5020 or 0.1% ethanol alone. Total RNA was isolated as previously described²². Aliquots (5 μg) were electrophoresed through horizontal, 1% agarose-formaldehyde gels, followed by transfer in 10 $\times$ SSC onto a Gene-scrim membrane (NEN), baking and UV-crosslinking²³. Hybridizations were performed as previously described²⁴. As probes, antisense cDNA transcripts²⁵ of rat ASS, rat CPS (both provided by S. Morris), rat GAPDH²⁶ and rat TAT²⁷ were used. Autoradiographs were exposed for 8–48 h.

FIG. 3 R5020 induces the same changes in the chromatin structure of the TAT gene as glucocorticoids. DNase I-hypersensitive sites flanking the TAT gene were mapped in clone 1 cells (a) expressing the chicken progesterone receptor and in the parental Fto2B-3 cells (b). Clone 1 cells and Fto2B-3 cells were treated with either 10 nM R5020, 100 nM dexamethasone (DEX) or no hormone (—). Nuclei were isolated and digested with different amounts of DNase I as indicated above each lane (numbers represent units DNase I per ml). DNA was isolated and digested with *Hind*III (H3). Resulting fragments were analysed by Southern blotting using the probe as indicated in (c). Hypersensitive sites (HS) are indicated by arrows. In addition to the glucocorticoid inducible hypersensitive site (HS III) two further hypersensitive regions which serve as internal controls are visible on the autoradiographs. These correspond to the previously mapped constitutive hypersensitive sites HS I and HS II in the 5' flanking region of the TAT gene^{12,28}. (Abbreviations: DEX, dexamethasone; HS, DNase I-hypersensitive site; M, Marker, *Hind*III-digested and end-labelled bacteriophage λ DNA.) Mapping of the DNase I-hypersensitive site was performed as described previously¹².

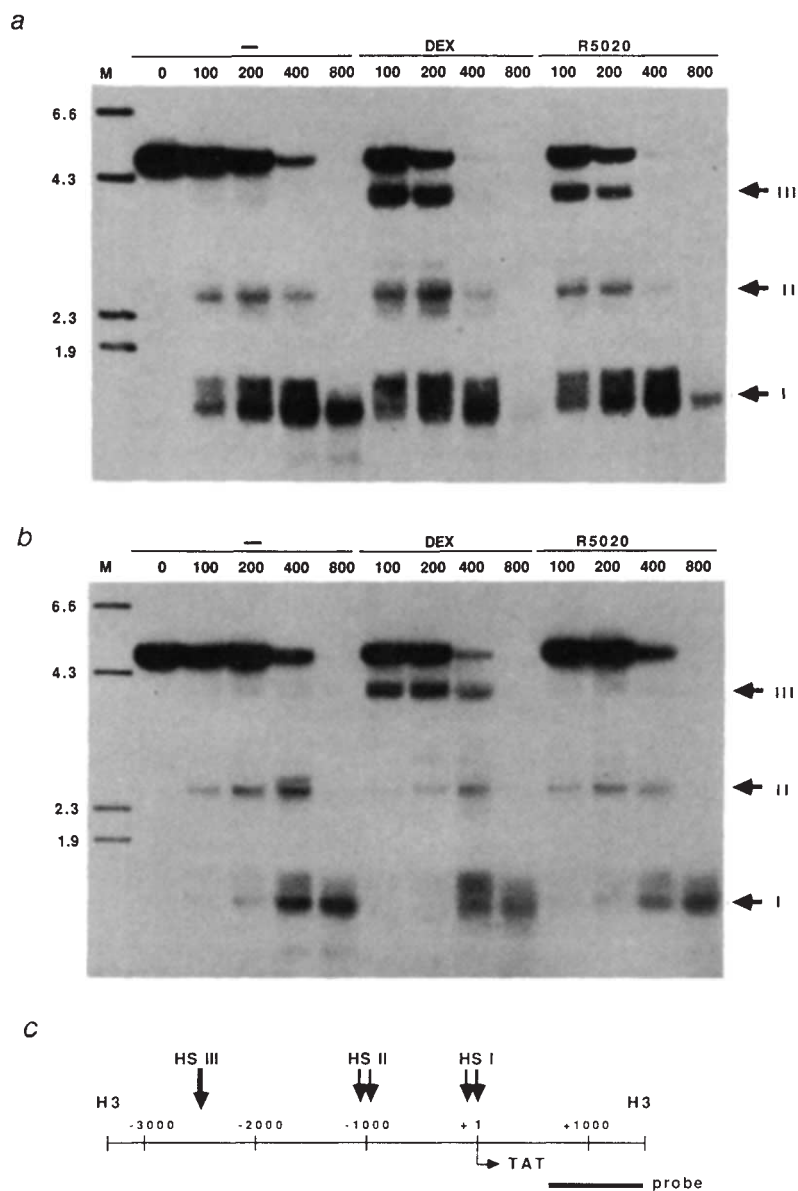
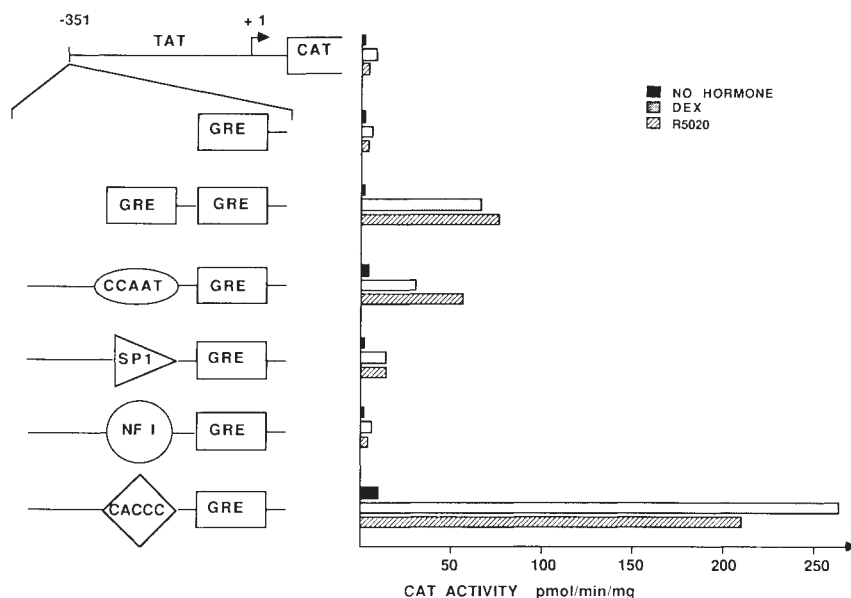


FIG. 4 Glucocorticoid receptor and progesterone receptor cooperate equally with other transcription factors. Constructs containing either one or two copies of the 15-bp GRE, or combinations of the GRE with a CCAAT-, CACCC-motif, the recognition sequence of SP1 or NF1 transcription factors upstream of the TAT gene promoter driving expression of the CAT gene, were transiently transfected into clone 1 cells. After electroporation, cells were split onto plates containing either 10 nM R5020, 100 nM dexamethasone or no hormone (0.1% ethanol alone). Induction of expression was followed by determining CAT activity in the extracts. Results are presented as pmol chloramphenicol acetylated per min and per mg of extract protein. For methods see Fig. 1. The plasmid constructs have been described elsewhere⁹.



Glucocorticoids induce the formation of a DNaseI-hypersensitive site 2.5 kilobases (kb) upstream of the transcription start site in the chromatin flanking the TAT gene in Fto2B-3 cells¹². This region binds the glucocorticoid receptor after hormone administration¹³ and is responsible for glucocorticoid inducibility of the TAT gene¹². The DNase I hypersensitive sites of the 5'-flanking region of the TAT gene were mapped in clone 1 and Fto2B-3 cells treated with dexamethasone or R5020. Both hormones induced the hypersensitive site (HS III), 2.5 kb upstream of the TAT gene, with equal effectiveness in clone 1 cells (Fig. 3a). Administration of R5020 to Fto2B-3 cells did not expose this hypersensitive site (Fig. 3b). Thus, the introduced progesterone receptor affects chromatin structure in the same way as the endogenous glucocorticoid receptor, implying that both receptors act through the same cis-regulatory elements.

Glucocorticoid and progesterone induction can be increased by cooperation of the receptors with other transcription factors^{9,14,15}. Synergism between the GRE and these other cis-regulatory elements is remarkably dependent on the cell line used to analyse the recombinant constructs, indicating that the activity of the factors recognizing these elements can vary considerably between different cell lines⁹. We therefore compared the inducibility of these constructs by R5020 and dexamethasone in clone 1 cells by monitoring CAT enzymatic activity after transient transfection. Details of the constructs are described in Fig. 4 (see also ref. 9). Concordant with our previous results⁹, a single 15 bp GRE did not confer inducibility on the TAT gene promoter when inserted 351 bp upstream of the transcription start site. Duplication of this GRE, or its combination with either a CACCC-, CCAAT- or SPI-sequence motif produced similar increases of CAT expression in response to either steroid hormone. No induction was detected with the construct containing a recognition sequence for nuclear factor I (NF I) combined with the GRE in this cell line. Thus, the two receptors showed a similar degree of synergism when the response elements were duplicated and both also cooperated with the same preference with other transcription factors.

In conclusion, several glucocorticoid-regulated genes in hepatoma cells respond to progestins in the same way as to glucocorticoids when the receptors for both steroid hormones are present. The progesterone receptor is expressed in only a few cell types⁷. It is not clear whether glucocorticoid receptor and progesterone receptor are both present in cells expressing genes that respond only to progesterone, such as the uteroglobin gene in rabbit uterus^{11,16}. The evidence presented here strongly suggests that simply the lack of the progesterone receptor in liver prevents progestins from inducing glucocorticoid-responsive genes. Hormone-specific gene activation in specialized target cells may, however, employ further mechanisms such as selective inactivation of the hormone, as has been suggested for glucocorticoids in aldosterone target tissues¹⁷, or differential gene activation by the A and B forms of progesterone receptor¹⁸. Nevertheless, our data imply that differential expression of progesterone receptor and glucocorticoid receptor is an important parameter in provoking the different physiological effects of these two classes of steroid hormones. □

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Alteration of mouse cytochrome P450_{coh} substrate specificity by mutation of a single amino-acid residue

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AS a family of structurally-related enzymes, cytochrome P450 (P450) monooxygenases exhibit paradoxical characteristics: although collectively the enzymes display a broad range of substrate specificities, individually they are characterized by a high degree of substrate and product selectivity. Mouse P450_{15α} and P450_{coh}, for example, which are expressed in female liver and male kidney cells^{1,2}, catalyse 15α-hydroxylation of Δ⁴ 3-ketone steroids, such as testosterone and 7-hydroxylation of coumarin, respectively³⁻⁶. In spite of their divergent catalytic activities, however, these enzymes differ by only 11 amino acids within their 494 residues⁵. To determine the structural basis of the different substrate specificities of P450_{15α} and P450_{coh} we therefore altered each of these 11 residues by site-directed mutagenesis, expressing the mutant cytochromes in COS-1 cells. We report that the activities of both cytochromes depend critically on the identities of the amino acids at positions 117, 209 and 365 and, moreover, that a single mutation in which Phe 209 is substituted by Leu is sufficient to convert the specificity of P450_{coh} from coumarin to steroid hydroxylation.

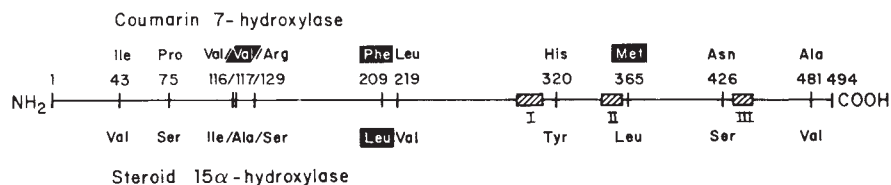
By altering each of the 11 substituted amino acids in P450_{coh} to the corresponding residue in P450_{15α}, we obtained a series of mutant enzymes denoted C1-11 (Fig. 1). Of these, C4, C6 and C9 exhibited only 35-45% of the coumarin 7-hydroxylase activity of wild-type P450_{coh} indicating that Val 117, Phe 209 and Met 365 all play important roles in the enzymatic function of the cytochrome (Table 1a). Moreover, the observation that a mutation as conservative as Val→Ala 117 profoundly affects the activity of P450_{coh} implies that Val 117 is located at a particularly critical position in the active site. When all three amino acids were changed in the same mutant (C469), the coumarin 7-hydroxylase activity was completely abolished, providing further proof that these three amino acids are essential for the maximal activity of wild-type P450_{coh}. Western blots performed to monitor the expression of wild-type P450_{coh} and its mutants, demonstrated that there was very little difference in the amounts of immunoreactive protein in the transfected cell homogenates (Fig. 2). It was concluded, therefore, that the changes in the activities of the mutants were not due to differences in the levels of expression in the transfected cells.

We also tested for increases in steroid 15α-hydroxylase activity in each mutant. As shown in Table 1a, only one mutant

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FIG. 1 Map showing the 11 amino-acid differences between P450_{coh} and P450_{15α}. Arabic numerals indicate the positions of the amino-acid residues. Amino acids found to be important in determining the substrate specificities of the cytochromes are indicated by the white letters on black backgrounds. Numbers I, II and III designate three conserved sequence motifs: the distal helix^{7,8}, the tridecapeptide¹⁸, and a peptide containing the Cys residue that forms a ligand to the haem, respectively¹⁹.



C6 (Phe → Leu 209) exhibited a significantly increased testosterone 15 α -hydroxylase activity which was ~100-fold greater than that of wild-type P450_{coh} and 70% that of wild-type P450_{15α}. Furthermore, we measured K_m values for testosterone 15 α -hydroxylation by wild-type P450_{15α} and mutant C6 and found that in both cases $K_m \approx 10 \mu\text{M}$. Similarly, we found the K_m values for coumarin hydroxylation to be in the same range (~1 μM) for both C6 and wild-type P450_{coh}. In addition to testosterone hydroxylation, mutant C6 catalysed 15 α -hydroxylation of progesterone and androstenedione (data not shown), showing that its steroid substrate specificity is identical to that of wild-type P450_{15α} (refs 4, 5). These results indicate that the Leu 209 is the most important determinant of steroid 15 α -hydroxylase activity, and that the Phe → Leu 209 mutation is sufficient to change the substrate specificity of P450_{coh} from coumarin to steroid hydroxylation. By comparison with C6, the mutant C469 exhibited a wild-type level of testosterone 15 α -hydroxylase activity, which implies that the occurrence of Ala and Leu at positions 117 and 365, respectively, is also important for maximum 15 α -hydroxylase activity.

Alteration of the corresponding amino acids in wild-type P450_{15α} confirmed the importance of Leu 209 to steroid 15 α -hydroxylase activity of the cytochrome. Mutant S6 showed a 100-fold decrease in activity (Table 1b) whereas by contrast S4 and S9 were 2–3 times more active than wild-type P450_{15α}. Wild-type P450_{15α} showed only a slight increase in coumarin 7-hydroxylase activity after altering Ala 117, Leu 209 and Leu 365 individually to the corresponding residues in wild-type P450_{coh}.

We previously reported that although coumarin is not catalysed by wild-type P450_{15α} it inhibits the steroid 15 α -hydroxylase activity of P450_{15α} to less than 10% of control levels⁶. By contrast, our present study shows that testosterone inhibits the coumarin 7-hydroxylase activity of P450_{coh}, but poorly (Fig. 3). It seems, therefore, that although coumarin is able to bind to the active site in P450_{15α}, testosterone is unable to bind to P450_{coh} whose steroid binding site, it must be concluded, is impaired by the replacement of Leu 209 with Phe. If this hypothesis is correct, testosterone should completely inhibit the coumarin 7-hydroxylase activity of C6, because this mutant cytochrome efficiently catalyses steroid 15 α -hydroxylase activity. As shown in Fig. 3, the C6-dependent activity was indeed almost completely inhibited by testosterone. In addition, double reciprocal plots of data obtained in the absence and presence of testosterone suggest that the steroid acts as a non-competitive inhibitor (data not shown). This is consistent with the hypothesis that coumarin and steroids bind to different adjacent sites in a catalytic domain that incorporates the amino acids at positions 117, 209 and 365.

It was intriguing to see that testosterone produced a sharp inhibition curve with a ~30% decrease in the coumarin 7-hydroxylase activity of wild-type P450_{coh}. Although the reason for this inhibition is uncertain, it is possible that P450_{coh} contains multiple coumarin-binding sites with different affinities which contribute differentially to the overall activity. It is then conceivable that testosterone, by binding at only one of these sites, does not fully inhibit the cytochrome's activity.

From the crystal structure of bacterial camphor hydroxylase (P450_{cam}) the distal helix, beginning at residue 248 and ending

TABLE 1 Hydroxylase activities of wild-type and mutant P450_{coh} and P450_{15α} cytochromes

a	Amino-acid residue		Coumarin 7-hydroxylase (pmol per min per mg protein)	Steroid 15 α -hydroxylase (pmol per min per mg protein)
	P450 _{coh}	P450 _{15α}		
C1	Ile 43 → Val 43		22.0	0.08
C2	Pro 75 → Ser 85		24.5	0.16
C3	Val 116 → Ile 116		20.7	0.06
C4	Val 117 → Ala 117		7.1	0.25
C5	Arg 129 → Ser 129		21.1	0.14
C6	Phe 209 → Leu 209		8.8	11.50
C7	Leu 219 → Val 219		20.0	0.09
C8	His 320 → Tyr 320		25.3	0.15
C9	Met 365 → Leu 365		9.8	0.12
C10	Asn 426 → Ser 426		22.1	0.13
C11	Ala 481 → Val 481		26.0	0.40
C469	Val 117, Phe 209, Met 365 → Ala 117, Leu 209, Leu 365		0.0	14.10
C0	Wild-type II		20.4	0.12
S0	Wild-type I		0.0	15.00
b				
S4	P450 _{15α} Ala 117 → P450 _{coh} Val 117		5.1	30.10
S6	Leu 209 → Phe 209		1.8	0.12
S9	Leu 365 → Met 365		1.3	49.80
S0	Wild-type I		0.0	15.00
C0	Wild-type II		20.4	0.12

Site-directed mutagenesis: Mutagenesis reactions were made with single-stranded complementary DNA templates in M13 vectors using the oligonucleotide-directed *in vitro* mutagenesis system (Amersham). The mutagenic oligonucleotide primers and the sequencing primers (for screening of the mutants) were prepared using the automated Beckman System 1 plus DNA Synthesizer. After synthesis, the primers were purified through a Sephadex G-25 (NAP-5 columns). After screening by sequencing with the dideoxynucleotide termination method¹³, the mutants were isolated from M13 vectors and ligated to the Okayama and Berg expression vector pcD. Expression in COS-1 cells: The recombinant plasmids containing the P450_{coh} (wild-type II) and P450_{15α} (wild-type I) cDNAs and mutant cDNAs were prepared by the alkaline lysis method followed by two CsCl centrifugations. Exponentially-growing COS-1 cells cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum were collected by trypsinization, replated at 1–2 × 10⁶ cells per 10-cm dish, and incubated for 24 h at 37 °C in 9 ml of the original media. The cells were washed with Dulbecco's phosphate-saline buffer and then transfected with a recombinant plasmid (3 μg per dish) in 500 $\mu\text{g ml}^{-1}$ of DEAE-Dextran for 30–60 min at 37 °C (ref. 14). The transfected cells were then treated with chloroquine (52 $\mu\text{g ml}^{-1}$) in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum for 5 h at 37 °C as described by Luthman and Magnusson¹⁵. After incubation with chloroquine, the medium was removed, the cells were washed with Dulbecco's phosphate-saline buffer, and then incubated for 72 h in 9 ml of the original medium. The cells were collected with a rubber policeman and suspended in 10 mM Tris-HCl buffer, pH 7.5, containing 150 mM KCl, 1 mM EDTA and 5 $\mu\text{g ml}^{-1}$ of leupeptin and pepstatin. The cell suspension was homogenized with Polytron, briefly sonicated and then used for measuring steroid 15 α -hydroxylase activity by the method of Harada and Negishi¹⁶ and coumarin 7-hydroxylase activity by the method described by Kaipainen *et al.*¹⁷. At least four different recombinant plasmids were transfected to COS-1 cells at the same time. The wild-type P450_{coh} cDNA was transfected for every experiment and activity levels were used to standardize the hydroxylase activities of the mutants. a, Alteration of amino-acids in P450_{coh} to the corresponding residues in P450_{15α}. b, Alteration of amino-acids in P450_{15α} to those in P450_{coh}.

at 259, was identified as the oxygen-binding pocket^{7,8}. Within this helix, Thr 251 is particularly notable in being conserved at the corresponding positions of all other P450s⁷. Moreover, recent studies, in which either site-directed mutagenesis of rabbit P450s⁹ or a naturally occurring yeast P450 mutant¹⁰ was used indicate the general importance of both the distal helix and the threonine in the catalytic activity. On the basis that P450_{coh} and P450_{15α} possess the same distal helix sequence, however, it seems that a modification of the helix would not affect the substrate specificities of the cytochromes.

Based on the X-ray crystal structure, Poulos *et al.* also suggested that a short helix, beginning and ending at residues 86 and 96 respectively, forms a substrate-binding pocket and discovered an active-site hydrogen bond between a substrate camphor and Tyr 96 in P450_{cam} (refs 7, 8). Recently, site-directed mutagenesis of P450_{cam} has demonstrated that this hydrogen bond regulates the affinity and specificity of camphor binding to the cytochrome^{11,12}. It is possible that a tyrosine at position 114 in P450_{coh} corresponds to Tyr 96 in P450_{cam} and forms a

hydrogen bond with the 2-ketone of coumarin, in which case it is probable that Val 117 determines the specificity of coumarin binding. At this stage, however, direct binding of coumarin to Val 117 cannot be ruled out. Conversely, the effect of residue 209 on the substrate specificity of P450_{15α} suggests that Leu at this position is necessary for steroid binding.

In conclusion, we have demonstrated that a single amino-acid mutation (Phe→Leu 209) is sufficient to alter the substrate specificity of P450_{coh} with the mutant cytochrome exhibiting the hydroxylase activity characteristic of P450_{15α} (low coumarin 7-hydroxylase activity and high steroid 15α-hydroxylase activity). Furthermore, although the structures of the active sites of P450_{coh} and P450_{15α} are likely to be similar it seems possible to account for their substrate specificities in terms of subtle differences between the contributions to substrate binding of the two different groups of residues at positions 117, 209 and 265. □

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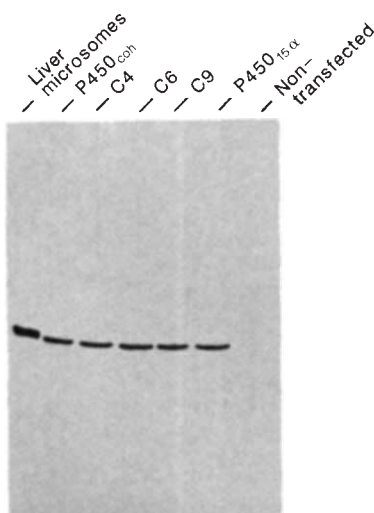


FIG. 2 Western blots of wild-type and mutant P450_{coh} and P450_{15α} proteins. The cell homogenates (12 μg) of COS-1 cells transfected with wild-type and mutant P450_{coh} cDNAs and P450_{15α} cDNA were electrophoresed on SDS-polyacrylamide gel (9%), transferred to nitrocellulose paper, and then immunostained with anti-P450_{15α} according to methods described previously⁵. Liver microsomes (1.5 μg) were prepared from 129/J male mice and used as a standard for the immunoreactive protein.

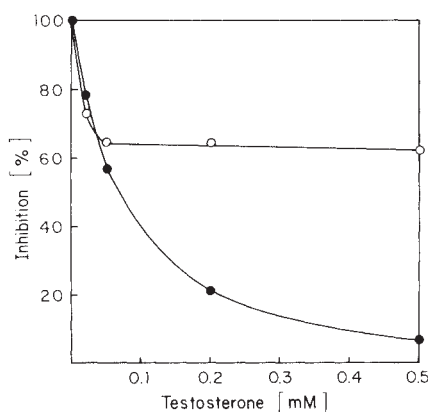


FIG. 3 Inhibition by testosterone of the coumarin 7-hydroxylase activity of the wild-type (○) and C6 mutant (●) P450_{coh}. The reaction mixtures were preincubated with various amounts of testosterone for 1 min at 37 °C, after which coumarin 7-hydroxylase reactions were started by addition of NADPH.

An unusual DNA structure detected in a telomeric sequence under superhelical stress and at low pH

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TELOMERIC sequences of DNA, which are found at the ends of linear chromosomes, have been attracting attention as potential sites for the formation of unusual DNA structures. They consist of (G_nT_m) or (G_nAT_m) motifs ($n \geq m$) and, in the single-stranded state, form hairpins stabilized by non-canonical G·G pairs¹. In the duplex state and under superhelical stress they exhibit hypersensitivity to SI nuclease² which by analogy with homopurine-homopyrimidine sequences^{3–5} may reflect the formation of an unusual structure. To determine whether this is the case we have inserted into a plasmid the *Tetrahymena* telomeric motif $(G_4T_2) \cdot (A_2C_4)$ and probed it by two-dimensional gel electrophoresis, chemical modification and oligonucleotide binding. Our data demonstrate that, under superhelical stress and at low pH, the insert does indeed adopt a novel DNA conformation. We

have concluded that in this structure the C-rich strand forms a hairpin stabilized by non-Watson-Crick base pairs $C \cdot C^+$ and $A \cdot A^+$, whereas the G-rich strand remains unstructured. We term this new DNA structure the (C, A)-hairpin.

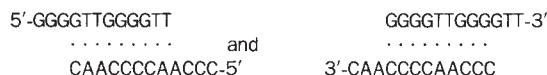
Figure 1 shows the two-dimensional gel electrophoresis patterns for our plasmid pTTEL3 carrying the insert $5'-G_3(T_2G_4)_7G_3(T_2G_4)_9$. At pH 4.4 a characteristic discontinuity is visible, which indicates that unlike the vector plasmid the plasmid carrying the insert undergoes a structural transition. A total superhelical release of approximately nine superhelical turns

was observed indicating that almost the entire insert of 102 base pairs (bp) forms an unusual structure and that in this structure the two complementary strands are not interwound. This conclusion was subsequently confirmed by chemical modification experiments (see below). The transition is strongly pH-dependent. Fig. 1 shows that, although the formation of an alternative structure is clearly seen at pH 4.4, no transition is observed at pH 5.0.

Thus, the insert under study exhibits a strongly pH-dependent structural transition which is similar to the transition inherent in

FIG. 1 Two-dimensional agarose gel electrophoresis of the pTTEL3 plasmid (a, b, d, e) carrying the insert $5'-G_3(T_2G_4)_7G_3(T_2G_4)_9$ and the vector pUC19 plasmid (c). Separation in the first directions (top to bottom): buffer, sodium citrate (40 mM) pH 5.0 (a and d) or 4.4 (b, c and e); electric field strength, 1.5 V cm^{-1} ; gel temperature, 10°C (a, b, c) or 30°C (d, e); time, 24 h. In the second direction (from left to right): buffer, TAE (40 mM Tris-acetate, pH 8.0, 1 mM EDTA); chloroquine ($1.7 \mu\text{g}$) was also added to relax superhelical stress. Note that although the patterns at 10° and 30° are qualitatively similar, there is a distinct and reproducible difference between them: the pattern at 10° exhibits a plateau after the mobility drop that is not observed at 30° .

METHODS. To tailor the pTTEL3 plasmid we used two oligonucleotides: $5'-\text{GGGGTTGGGGTT}$ and $5'-\text{CCCAACCCCAAC}$. On annealing, two types of structures can form:



The self-ligation of these blocks leads to the formation of long duplex repeats of the type $(G_4T_2)_n$ and we used this process to obtain fragments of about 100 bp. The 3'-retarded ends were then elongated with the Klenow fragment of DNA polymerase I and the resulting fragments inserted in the *Sma*I site of the pUC19 polylinker. These plasmids were used to transform *E. coli* JM83 cells. Maxam-Gilbert sequencing of the resulting pTTEL3 plasmid showed that it carried the insert $G_3(T_2G_4)_7G_3(T_2G_4)_9$.

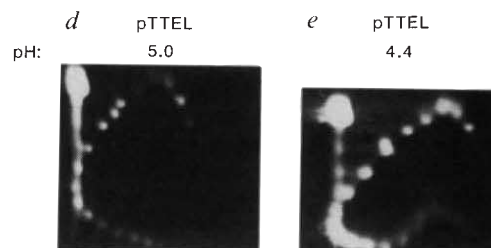
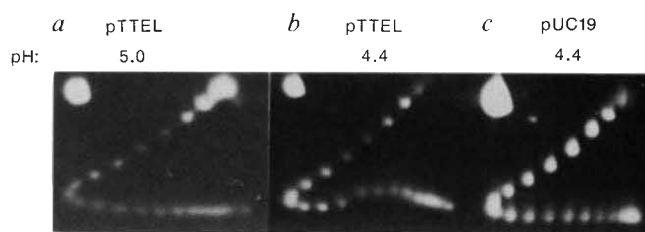
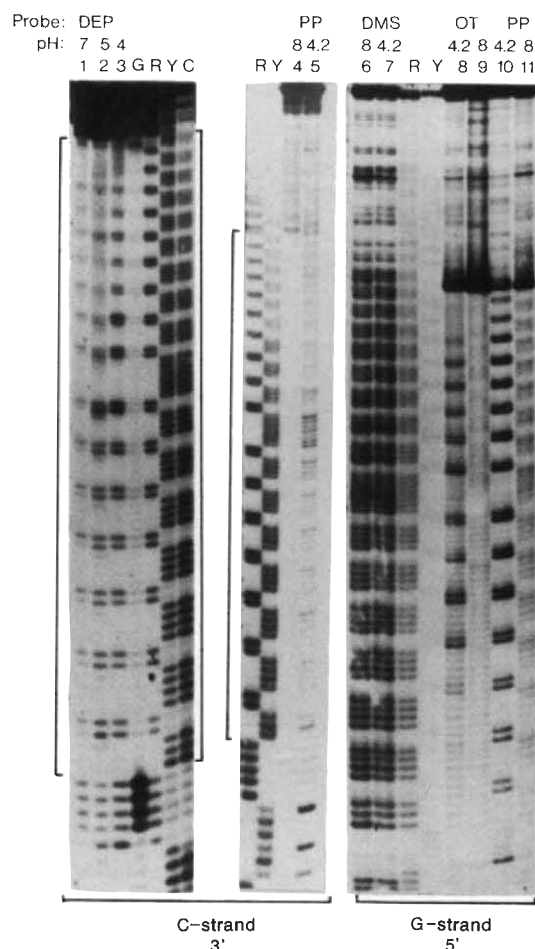


FIG. 2 The chemical probing of the $G_3(T_2G_4)_7G_3(T_2G_4)_9$ insert (indicated by brackets) within the pTTEL3 plasmid. For the C-strand, the 5'-end is at the top, whereas for the G-strand it is at the bottom. Lanes R, Y, C and G are standard Maxam-Gilbert probes for purines, pyrimidines, cytosines and guanines, respectively. Lanes 1, 2 and 3 show the results of diethyl pyrocarbonate (DEP) modification at pH 7.0, 5.0 and 4.0, respectively. Lanes 4, 5, 10 and 11 show the effects of potassium permanganate (PP) modification at pH 4.2 (lanes 5, 10) and 8.0 (lanes 4, 11). Lanes 6 and 7 show the results of dimethylsulphate (DMS) modification at pH 8.0 and 4.2 respectively. Lanes 8 and 9 show the effects of modification with osmium tetroxide (OT) at pH 4.2 and 8.0 respectively.

METHODS. DEP modification was performed as described by Voloshin *et al.*⁹. At pH 8.0 Tris HCl buffer was used, whereas at pH 5.0 and 4.0 a Na-acetate buffer was used. DMS modification was performed in 200 ml of a solution containing 3–5 μg plasmid DNA, 1 μl DMS, 1 mM EDTA, and either 50 mM Na-acetate, pH 4.2 or a buffer consisting of 20 mM Tris HCl, 50 mM Cl, pH 8.0; incubation time, 1 min at 10°C . The reaction was terminated by the standard DMS-stop solution with subsequent ethanol precipitation. OT modification was performed in 50 μl of a solution containing DNA, 1 mM EDTA, 4 mM OsO_4 and either 50 mM Na-acetate plus 2% pyridine, pH 4.2 (titrated by acetic acid) or a buffer containing 20 mM Tris HCl, 50 mM NaCl plus 2% pyridine, pH 8.0; incubation time, 60 min at 10°C . The reaction was terminated by ethanol precipitation. PP modification was performed in 50 μl of a solution containing DNA, 1 mM EDTA, 100 μM KMnO_4 and either 50 mM Na-acetate, pH 4.2 or 20 mM Tris HCl plus 50 mM NaCl, pH 8.0; incubation time, 5 min (pH 4.2) and 20 min (pH 8.0) at 10°C . The reaction was terminated by ethanol precipitation. Substitution of 1–5 mM MgCl_2 for EDTA did not change the patterns. Similar modification patterns were observed at 30° .



homopurine-homopyrimidine palindromes⁵⁻⁸. This raises the question of whether the alternative structure present in the pTTEL3 plasmid is the triplex H form. This is doubtful, however, because the sequence deviates considerably from the homopurine-homopyrimidine pattern and even isolated nucleotide substitutions are known to seriously inhibit the formation of the triplex H form⁷.

The most convincing evidence of the triplex H form in homopurine-homopyrimidine palindromes has been obtained by chemical probing⁹⁻¹⁶. Therefore, we applied this method to the pTTEL3 plasmid using: diethyl pyrocarbonate (DEP), which modifies adenines in the single-stranded and Z-form state, but not in B-form and triplex states; dimethylsulphate (DMS), which modifies guanines in all conformations except the triplex; osmium tetroxide (OT) and potassium permanganate (PP), which modify only pyrimidines in the single-stranded state. The results, shown in Fig. 2, indicate that the pattern of modification is different from that of the triplex H form⁹⁻¹⁶. Indeed, in the present case all chemical probes show symmetrical modification patterns (within the insert) for both strands. The two strands nevertheless are modified quite differently. The modification of the G-rich strand is more or less uniform throughout the insert. At pH 4.2 all the thymines of the insert are accessible for reactions with both OT and PP, and there is no protection of guanines against the reaction with DMS. These data indicate that within the structure the bases of the G-rich strand are virtually unpaired.

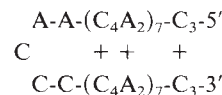
By contrast, apart from a distinctive modification of the 3'-end-flanking region of the C-rich strand of the insert only the central part of this strand is heavily modified at pH 4.2. Our chemical probing data confirm that the structure of the insert is stabilized by low pH (see Fig. 2).

We believe that our data are most consistent with the structure in which the two strands form independent hairpins with non-canonical base pairing. Specifically, the C-rich hairpin is stabilized by the protonated C·C⁺ base pairs and this explains why this structure, which we term the (C, A)-hairpin (Fig. 3), is strongly pH-dependent.

The (C, A)-hairpin is stabilized primarily by the antiparallel C·C⁺ base pairs¹⁷. But the DEP modification pattern is also consistent with the formation of A·A⁺ base pairs. Although several protonated pairing schemes of A·A⁺ base pairs have been proposed (see ref. 18), we were unable to find in the

literature an A·A⁺ pair isomorphous to the C·C⁺ pair. We propose a pairing scheme in which the N1-protonated adenine forms two hydrogen bonds with the unprotonated one: N7··H₂N and C8··N1. We believe that the protonation in the N1 site makes possible the formation of the unorthodox hydrogen bond between C8 of the protonated adenine and N1 of the unprotonated one. Such A·A⁺ pairs are virtually isomorphous to the antiparallel C·C⁺ pairs.

The detailed pairing scheme for the C-rich hairpin, which is consistent with the chemical modification data and two-dimensional gel electrophoresis, is as follows:



Although the three central A₂ sites are the most DEP-accessible in the structure (Fig. 2, lane 3), the other A₂ sites are also reactive. This is concordant with the proposed pairing scheme for A·A⁺, in which one of the two N7 positions in the pair may be accessible for modification. Indeed, it is known that, by contrast with B-DNA, in Z-DNA the adenine N7 position is accessible to DEP¹⁹⁻²¹. From Fig. 2, lane 3 it is clear that four guanines and one adenine in the 3'-flanking region are also modified by DEP, which may reflect stable or transient unwinding of the duplex from its open end under superhelical stress. A similar effect was observed in the case of DEP-modification of the H form⁹⁻¹⁶.

Chemical probing failed to reveal any structure in the G-rich hairpin: this was unexpected because the formation of antiparallel G·G base pairs in single-stranded telomeric sequences had been demonstrated previously¹. One explanation for these conflicting results is that the G·G base pairs are destabilized under mildly acid conditions.

The asymmetry of the two strands in the (C, A)-hairpin was additionally tested by investigating complex formation between the C₃A₂C₄A₂C (C-rich) and (G₄T₂)₂ (G-rich) oligonucleotides

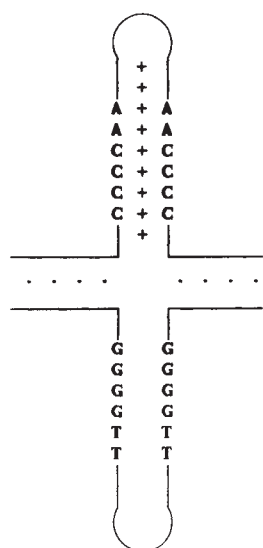


FIG. 3 The proposed model of the (C, A)-hairpin is stabilized by the C·C⁺ and A·A⁺ base pairs. Our data indicate that the G-rich strand is virtually unstructured. The structure is observed at pH < 4.5 under superhelical stress.

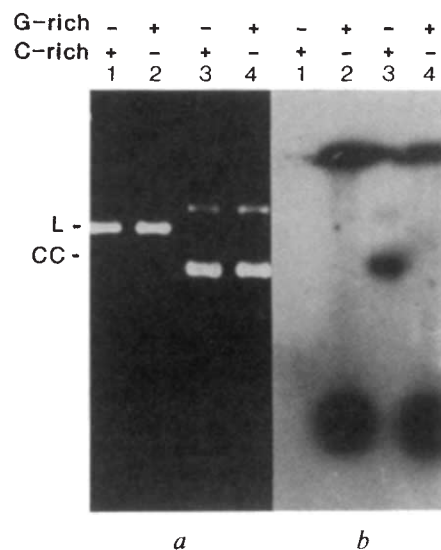


FIG. 4 Agarose gel electrophoresis of linear (lanes 1, 2) and supercoiled (lanes 3, 4) plasmid pTTEL3 DNA. The samples were preincubated for 6 h in the Na-citrate buffer (100 mM pH 4.5) with the deoxioligonucleotides 5'-CCCAACCCCAAC (C-rich, lanes 1, 3) and 5'-GGGGTTGGGGTT (G-rich, lanes 2, 4). γ -³²P-ATP-labelled by polynucleotide kinase of phage T4. The C-rich oligonucleotide concentration was ~0.5 μ g/ml, with which DNA was approximately equimolar. Electrophoresis was performed for 15 h in 2% agarose gel, 100 mM Na-citrate buffer (100 mM of Na⁺, pH 4.5), with an electric field strength of 1.2 V/cm, and a gel temperature of 10 °C. Linear and closed circular DNA are labelled L and CC, respectively. Panel a, ethidium stained gel. Panel b, autoradiograph of the gel in panel a. The upper band on lanes 3 and 4 in panel a contains closed circular dimers of the pTTEL3 plasmid.

and plasmid pTTEL3 DNA. Figure 4 shows the results: although neither oligonucleotide binds to non-superhelical DNA, $C_3A_2C_4A_2C$ forms a complex with superhelical DNA at low pH, which was revealed as a strong band in lane 3 in Fig. 4b. This band was not observed at pH 8 (data not shown). Moreover, with $(G_4T_2)_2$ no trace of radioactivity was observed to move with DNA at either pH 4.5 or pH 8 (Fig. 4b, lane 4), even in the presence of an excess of the G-rich oligonucleotide (compare lanes 1 and 2 in Fig. 4b). These data support our model of the (C, A)-hairpin in which a stable C-rich hairpin and virtually unstructured G-rich strand allow the uptake of an oligonucleotide complementary to the latter but not the former. The results shown in Fig. 4 are also significantly different from those obtained when complexing homopurine and homopyrimidine oligonucleotides with a plasmid carrying the homopurine-homopyrimidine insert²².

Note that although the data we present support the (C, A)-hairpin model, they are not absolutely unequivocal. Specifically, we cannot completely exclude the possibility of formation of the triplex H form by our insert which carries, along with the canonical TAT and CGC⁺ triads, non-canonical base-triads, such as CGA and ATC.

Binding of $(G_4T_2)_2$ probes to the single-stranded G-rich part of the (C, A)-hairpin was not observed, although, on the basis of previous observations²³, parallel four-stranded complexes might be expected to form. Again, one possible explanation could be the destabilizing effect of low pH on the G_4 tetrads. It should also be noted, however, that in our experiments the oligonucleotide concentration was approximately 100-fold lower than that used in the previous study and that conditions were therefore not conducive to the formation of a multi-stranded complex.

It follows from our model that the sequence requirement for the (C, A)-hairpin is a poly(A, C)·poly(G, T) mirror repeat which we label as an (A, C)-palindrome. It thus remains to unequivocally demonstrate this requirement in the same way that the H palindrome was shown to be the sequence requirement of the H form⁷.

Finally, it is reasonable to suppose that the ability of telomeric sequences to adopt the (C, A)-hairpin structure under superhelical stress is biologically significant: not only do telomeric sequences exist predominantly in the single-stranded state at the ends of chromosomes but similar sequences have also been found within chromosomes^{24,25}. □

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Triple-strand formation in the homopurine:homopyrimidine DNA oligonucleotides $d(G-A)_4$ and $d(T-C)_4$

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INTEREST in triple and quadruple DNA helices built from homopurine and homopyrimidine strands has recently intensified principally because such structures may occur *in vivo*¹ but also because of the potential use of triplexes both in forming highly sequence-specific complexes for use in chromosome mapping^{2,3} and in repressing transcription⁴. From fibre diffraction data, models for triplex structures with poly(U)·poly(A)·poly(U) and poly(dT)·poly(dA)·poly(dT) have been proposed⁵, in which the purine and one pyrimidine strand are Watson-Crick paired in an A' helix, and the other pyrimidine strand is Hoogsteen base-paired parallel to the purine strand along the major groove. A similar base-pairing scheme involving G and C would require protonation of C for Hoogsteen base-pair formation, and models for such triplexes have been proposed^{1,6} by analogy to the single-sequence fibre diffraction data. To date, however, there have been no single crystal or NMR structural data on DNA triplexes, and no direct observation of the protonated C in such a context. We present here the first NMR evidence for triplex formation in DNA from the homopurine $d(G-A)_4$ and homopyrimidine $d(T-C)_4$ oligonucleotides, and report direct observation of imino protons from protonated cytosines in the triplex.

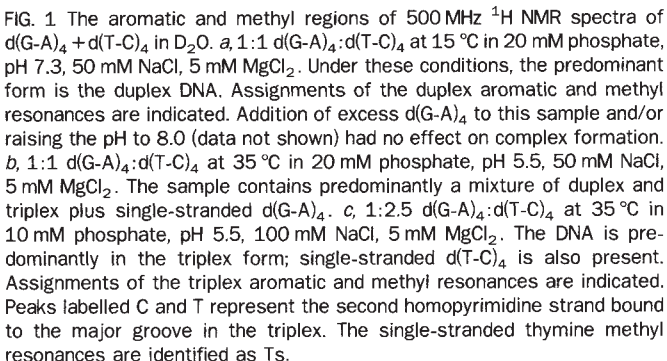
Runs of $(dT-dC)_n$ · $(dA-dG)_n$ are found at unexpectedly high levels in eukaryotic genomes¹. Such sequences show a hypersensitivity to DNase 1 and single strand-specific nucleases that is dependent on pH and supercoil density, which indicates that they are adopting an unusual structure^{7–13}. At present the most widely accepted model for this structure is that of Frank-Kamenetskii and co-workers in which the homopurine:homopyrimidine sequence loops out to form a pyrimidine·purine·pyrimidine triplex and a single-stranded homopurine stretch¹⁴. This model has been supported and expanded on by several recent studies^{15–17}.

NMR spectra of the aromatic and methyl resonances of the complexes formed from $d(G-A)_4$ and $d(T-C)_4$ under different conditions are shown in Fig. 1. Figure 1a shows the spectrum observed at pH 7.3 (50 mM NaCl, 5 mM $MgCl_2$) with equal amounts of $d(T-C)_4$ and $d(G-A)_4$ strand present. Under these conditions the predominant structure is the DNA duplex $d(G-A)_4$ · $d(T-C)_4$, and NOESY¹⁸ experiments gave spectra with crosspeak patterns typical of a regular B-DNA helix (spectrum not shown). Assignments for all non-exchangeable protons (except H4', H5', H5'') were obtained for this structure by analysis of COSY¹⁹, DQF-COSY²⁰ and NOESY spectra using the sequential assignment techniques that are now standard for B-DNA-type structures^{21–23}. Assignments of the aromatic and methyl proton resonances in the DNA duplex are as indicated in Fig. 1. Dramatic changes were observed when the pH of the sample was lowered to 5.5: new resonances were evident throughout the aromatic and methyl regions and were most obvious between 6.7 and 7.5 p.p.m. Our analysis of this sample, discussed below, showed that under these conditions it contains a mixture of the B-DNA duplex and a triplex composed of one $d(G-A)_4$ and two $d(T-C)_4$ strands, as well as some single-stranded $d(G-A)_4$. Addition of excess $d(T-C)_4$ strand pushes the equilibrium towards the triplex form (Fig. 1c) and under

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An examination of the assignments shown in Fig. 1c reveals some unusual chemical shifts for resonances in the triplex. All of the base resonances in the homopurine strand are shifted upfield from their resonance positions in duplex DNA, with three of the adenines showing shifts greater than 1 p.p.m. The resonances from the second d(T-C)₄ strand are all shifted downfield relative to their positions in a duplex or a single strand. We also note the appearance of some small unidentified resonances in the aromatic region of the duplex spectrum. Resonances at 7.17 and 8.13 p.p.m., for example, in Fig. 1a which show exchange crosspeaks with both strands of the duplex DNA, but do not correspond to the resonances identified from the triplex. They appear at temperatures up to 35 °C and in the pH range 6.1–8.3 in the absence and presence of magnesium. Addi-

Figure 2 shows spectra of the DNA complexes in H₂O. Figure 2a shows the H₂O spectrum of the imino and amino resonances recorded under conditions where the predominant conformation is a B-DNA duplex. At 5 °C, the imino proton resonances from the eight Watson-Crick A · T and G · C base pairs can be readily identified. Figure 2b shows the same spectral region for the sample under the conditions described in Fig. 1c, where the predominant conformation is a DNA triplex. As in the case of the D₂O spectra, the triplex spectra are significantly different from those of the duplex and contain several additional imino and amino proton resonances. By analysis of NOESY spectra these new imino resonances have been identified as arising from



METHODS. Spectra were obtained from 64-128 transients acquired over 8K complex points with a sweep width of 5,000 Hz and 2-s recycle delay. The spectra were resolution enhanced by double exponential multiplication. All of the spectra were acquired on a GN 500 MHz spectrometer and processed on a VAX 8800 or Microvax II using the software package FTNMR (Hare Research). The DNA oligonucleotides were synthesized by the phosphotriester method and purified in the laboratory of Dr Jacques van Boom as previously described³⁰ or synthesized on an Applied Biosystems 381A DNA synthesizer using phosphoramidites and a 10- μ mol column. The latter DNA was purified by gel filtration following the method of Kintanar *et al.*³¹ Purified oligonucleotides ran as single bands on denaturing polyacrylamide gels. Sample concentrations were determined from A_{260} measurements using extinction coefficients of 9,500 1 mole⁻¹ cm⁻¹ and 7,500 1 mole⁻¹ cm⁻¹ for d(G-A)₄ and d(T-C)₄, respectively^{6,32}, and were 2-6 mM in each strand depending on the sample.

Hoogsteen base pairs (Fig. 3 and spectra not shown; ref. 34): Watson-Crick A·T base pairs are identified by a strong imino-AH2 crosspeak²⁷, whereas Hoogsteen A·T and G·C base pairs show a strong crosspeak between their imino and H8 resonances²⁸. The imino protons from the Hoogsteen G·C base pairs thus identified resonate at around 15.4 p.p.m., which is approximately 3 p.p.m. downfield from the resonance positions of Watson-Crick base-paired G·C imino protons. Hoogsteen base-pairing of G·C requires protonation of the C (Fig. 4). Although this base-pairing scheme has been proposed in triplex models containing G and C (refs 1, 2, 6), identification of these imino resonances represents the first direct observation of the protonated dC in DNA triplexes and therefore confirms that aspect of the models. Several unusually lowfield-shifted amino resonances (8.5–10 p.p.m.) are also present in the spectrum of the triplex, which give rise to strong crosspeaks with the C⁺·G imino resonances and have been identified as the hydrogen- and non-hydrogen-bonded C⁺ amino protons. The requirement for protonation of the C for Hoogsteen G·C base-pair formation also explains the dependence of triplex formation on pH.

A portion of the NOESY spectrum of the triplex in H₂O containing the imino resonances and their crosspeaks is shown in Fig. 3a. In current models of pyrimidine·purine·pyrimidine triplexes, the third strand is Hoogsteen base-paired in the major groove parallel to the homopurine strand and the Watson-Crick base-paired strands are in an A'-DNA-like conformation². Binding of the third strand in the major groove by Hoogsteen base-pairing should result in a set of imino protons that show sequential NOE connectivities along the strand. Parallel binding of the

second d(T-C)₄ strand would result in a one-base offset of this strand, giving rise to either four C⁺·G·C and three T·A·T base triplets or three C⁺·G·C and four T·A·T base triplets, depending on whether the extra base is at the 3' or the 5' end (Fig. 3b). We have identified four T·A and three C⁺·G Hoogsteen-paired imino resonances which can be sequentially assigned in the NOESY spectrum. Thus, the major form of the triplex present has a 3' dangling C and is in equilibrium with a small amount of a triplex containing a 5' dangling T (ref. 34). We suggest that the predominance of the 3' dangling C triplex is due to the greater stability of A·T versus G·C⁺ Hoogsteen base pairs. This will be important in designing sequence-specific probes for chromosome mapping^{2,3}.

We have also investigated the sugar conformations in the triplex. In models based on fibre diffraction of dT·dA·dT, all

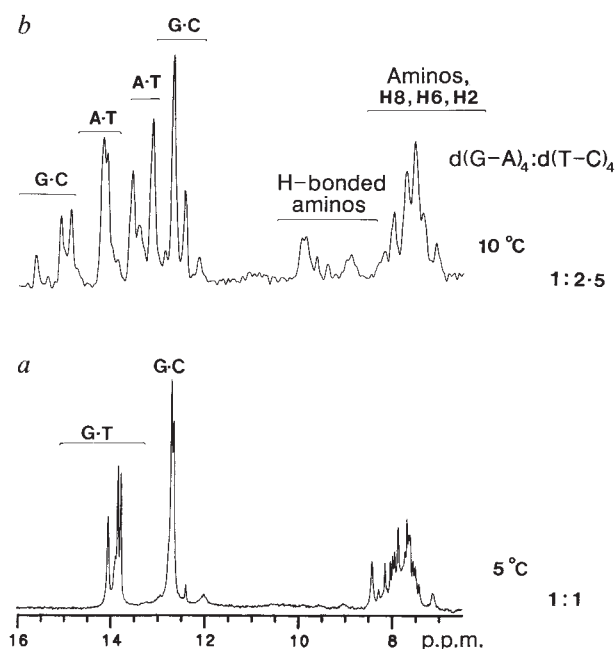


FIG. 2. The imino, amino, and aromatic regions of 500 MHz ¹H NMR spectra of d(G-A)₄:d(T-C)₄ under different conditions in 90% H₂O, 10% D₂O. *a*, 1:1 d(G-A)₄:d(T-C)₄ at 5°C in 20 mM phosphate, pH 7.3, 50 mM NaCl, 5 mM MgCl₂ (same sample as in Fig. 1*a*). Watson-Crick base-paired A·T and G·C imino resonances are identified. *b*, 1:2.5 d(G-A)₄:d(T-C)₄ at 10°C in 10 mM phosphate, pH 5.5, 100 mM NaCl, 5 mM MgCl₂ (same sample as Fig. 1*c*). Formation of the triplex is indicated by the presence of resonances arising from Hoogsteen (*) base pairs, as well as additional hydrogen-bonded amino resonances. The spectra were obtained using a 11 spin-echo (90°_x-τ-90°_x-Δ-90°_φ-2τ-90°_φ) pulse sequence³³. The delay τ was adjusted so that the region of maximum excitation was centred between the aromatic and the imino proton resonances. The spectra were acquired over 8K complex points with a sweep width of 14,048 Hz and line-broadened by 3 Hz before Fourier transformation.

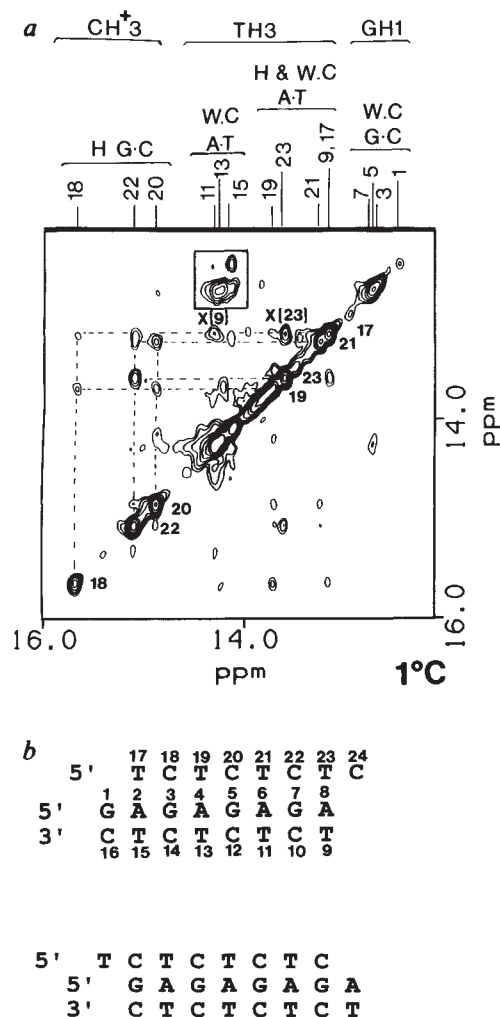


FIG. 3. *a*, Portion of the NOESY spectrum of the triplex in 90% H₂O, 10% D₂O at 1°C showing the region containing the imino proton resonances and their crosspeaks. Sample conditions were the same as Fig. 2*b*. Assignments for the Watson-Crick and Hoogsteen base pairs are shown and sequential assignment of the Hoogsteen base-paired iminos are indicated by the dotted lines. Additional crosspeaks, arising from exchange between the two triplex forms (*b*), are labelled x. The phase sensitive spectrum was obtained by replacing the last 90° pulse in the standard NOESY pulse scheme with a 11 spin-echo pulse sequence³³ (90°_x-τ-90°_x-Δ-90°_φ-2τ-90°_φ). *b*, Schematic of the two triplex forms with the numbering system used. The triplex at top is the predominant form observed.

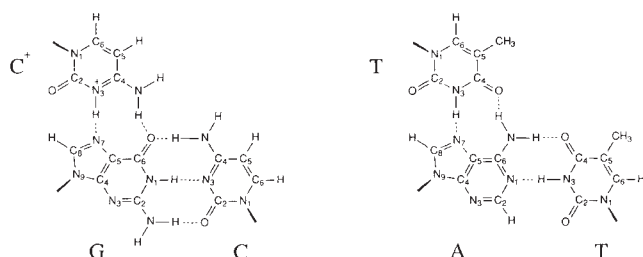


FIG. 4. $C^+ \cdot G \cdot C$ and $T \cdot A \cdot T$ base-pairing scheme in the triplex.

three strands are in an A' conformation in which all the sugars are C3'-endo. The results of NOESY and MINSY²⁹ experiments (data not shown), however, indicate that although both homopyrimidine strands have an N-type sugar pucker, close to C3'-endo, the purine strand does not. It seems therefore that either the conformation of mixed base purine·pyrimidine·pyrimidine triplexes is somewhat different from dT·dA·dT triplexes or the fibre diffraction model is not completely correct.

To our knowledge, this is the first NMR evidence for triplex formation between homopurine and homopyrimidine DNA strands. We have shown that d(G-A)₄ and d(T-C)₄ will, under appropriate conditions, form a d(T-C)₄·d(G-A)₄·d(C⁺-T)₄ triplex in which the first two strands are Watson-Crick base-paired and the second homopyrimidine strand is Hoogsteen base-paired in the major groove to the homopurine strand. Triplex formation is favoured by low pH and addition of excess d(T-C)₄ strand. We find no evidence for formation of a pyrimidine·purine·purine triplex, but do see indications that a third, as yet unidentified, heterocomplex is present under some conditions. □

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High-performance capillary electrophoresis

B.L. Karger

High performance capillary electrophoresis is expected to be the fastest-growing analytical technique since HPLC. The method has already shown its utility in a variety of applications.

High-performance capillary electrophoresis (HPCE) is undergoing rapid development at the present time^{1,4}. This instrumental approach to electrophoresis offers great potential in resolving power, rapid separation, automation and quantitation. The degree of interest in the field is comparable to that expressed for high performance liquid chromatography (HPLC) in the early 1970s. Recently, the First International Symposium on HPCE was held in Boston, Massachusetts, and a second symposium will be held 29–31 January 1990 in San Francisco, California.

Historically, electrophoresis was first performed in tubes, but this format became less important as slab gels were developed. Nevertheless, even today columns are often used for the isoelectric focusing first dimension of 2-dimensional electrophoresis. But unlike tube gels, HPCE offers the capability to detect samples on-line (or on-column) as in column chromatography. Spectroscopic detection is accomplished by using fused-silica capillaries and removing a small section of polyimide near one end of the capillary. Exposure of the fused silica tubing allows UV or fluorescence detection on-column.

In electrophoresis it is well-known that high electric fields yield fast separations. In the case of capillary electrophoresis, when injection and detector volume effects are minimized, as well as such factors as adsorption on the walls of the capillary, the major band-broadening mechanism is that of axial diffusion¹. The faster the species migrates through the capillary, the less time is available for diffusion, and hence, the sharper the bands produced. Thus, high electric fields (typically 100–300 V cm⁻¹) will yield high efficiency along with rapid separation. Indeed, it is possible to generate routinely 3–5 × 10⁵ theoretical plates⁵ and, with care, even several million plates for column lengths of one metre or less. Capillaries of roughly 25–100 µm in diameter are employed to minimize the Joule heat due to the high electric fields. If not minimized or removed, the heat can cause band broadening and changes in column temperature.

There are currently six manufacturers of full HPCE equipment (Applied Biosystems, Beckman Instruments, Bio-Rad, Dionex, Shimadzu and SpectroVision). Isco and Linear Instruments offer UV detectors for HPCE, and SCIEX has recently introduced an HPCE interface for its mass spectrometer. Most basic

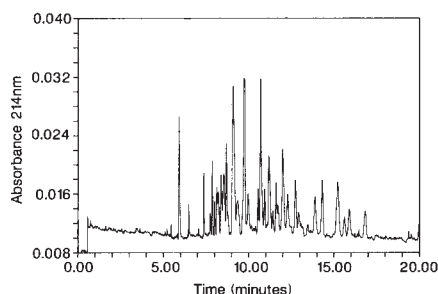


FIG. 1 Tryptic digest HPCE map of bovine serum albumin. Conditions: 330 V cm⁻¹, 72 µA; effective length 50 cm, total length 60 cm. Buffer: 20 mM Na₂H₂PO₄, 1.5 M urea, pH 3.0.

setups consist of a power supply, generally in the range of 30–60 kV, and a UV or fluorescence on-line detector. A safety system is included on the high voltage side of the instrument to break the circuit before it comes in contact with the user. Injection is accomplished either by electrophoresis or by pressure, typically using an autosampler. The detector signal is processed in a manner similar to HPLC. While no commercial instrument now offers an automated fraction collector, this component may be available soon.

The proper detection system is important to the success of any HPCE experiment. The mass sensitivity of UV and fluorescence detectors used in HPCE at the present time is often two to three orders of magnitude lower than that of conventional HPLC detectors. But the smaller cell volume means that the concentration sensitivity of HPCE is often comparable to HPLC. Other detectors which are likely to be used with HPCE are laser-induced fluorescence detectors, with attomolar and subattomolar detection limits^{6,7}; electrochemical detectors⁸; and mass spectrometers⁹. In the latter case, on-line coupling is generally accomplished at atmospheric pressure, using electrospray ionization^{10,11}. There is no question that on-line HPCE mass spectrometry will be fully utilized in the years ahead.

A variety of separation principles have been developed using capillary electrophoresis, either in the open-tube or gel-filled format. In open-tube operation, one approach involves the use of electroosmosis as a bulk-flow medium in which the zeta potential (typically negative in the case of fused silica) is used to create bulk flow. With fused silica, positive species migrate faster, and negative species slower than the bulk flow, while neutrals move with the bulk flow.

The open-tube approach has been found to be fully complementary to HPLC (reversed-phase LC) in the separation of peptides and proteins. The use of HPCE for peptide mapping, as illustrated in Fig. 1 for bovine serum albumin¹², is readily achievable, using either pH 2–3 phosphate buffer or pH 8–9 buffer^{13,14}. There is no correlation between the elution order of the peptide map in reversed phase LC and that in HPCE, and thus different analytical information is obtained from the two methods. HPCE may also be used for the determination of peptide/protein variants, such as deamidation^{13,14} and incorrectly folded species¹⁵, and the open-tube approach can be used in oligonucleotide restriction fragment mapping, where a wide molecular weight range can be separated in one run⁵. Many species traditionally examined by HPLC, such as drugs, hormones and chiral species, can be explored with HPCE as well. The method provides reproducible analysis, with migration times differing by 0.5 per cent or less, which complements HPLC.

Many different chemistries can be used with the open-tube format of HPCE. A particularly good example is the use of SDS-micelles for the separation of neutral and charged species^{16,17}. In the micellar electrokinetic approach, neutral species is partitioned between the hydrophobic interior of the micelle and the bulk solution. Because the micelle is dragged at a slow fixed rate by electroosmotic flow toward the negative electrode, electrophoretic separation is transformed to differences in partition, with the micelle considered equivalent to the stationary phase in chromatography. The surface of the micelle can also be used, either by adsorbing metal to its surface and separating by ligand exchange¹⁸, or by ion-pair interaction with the charged surface¹⁹.

Isoelectric focusing offers yet another separation approach in HPCE. In this technique, the walls of the capillary are

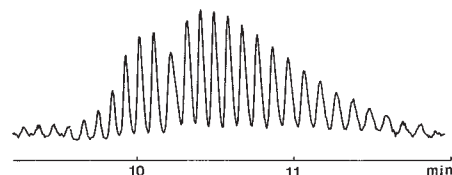


FIG. 2 Capillary polyacrylamide gel separation of polyadenylic acid 40–60-mers. Conditions: 300 V cm⁻¹, 11 µA; effective length 15 cm, total length 35 cm; 6 per cent T, 5 per cent C. Buffer: 0.1 M Tris, 0.25 M borate, 7 M urea, pH 8.3.

coated with a non-charged polymer to minimize electroosmosis, and the sample is mixed with carrier ampholyte. The mixture is placed in the capillary and focused, as determined by the drop in current to a low or zero value. The sample is then forced to migrate past the detector, either by pressure or by the addition of salt to the catholyte or anolyte²⁰. In this manner, separation based on pI is achievable. The method can also separate isoforms²¹.

Polyacrylamide gels can be polymerized in the capillary for HPCE based mainly on size differences. SDS-gel electrophoresis is possible²², and oligonucleotides can be separated with high efficiency using the capillary gel format. Fig. 2 shows the separation of a mixture of 40–60-mers of polyadenylic acids²³. The high efficiency and speed of this approach makes it useful as a means to assess the purity of oligonucleotides produced by a DNA synthesizer. The method also has great potential in DNA sequencing.

HPCE is not simply an analytical tool. It can be used for the collection of picomole amounts of material for microsequencing and other methods of characterization. Collection is relatively straightforward, and can be accomplished in water. Even for complex chromatograms, the sample can be run using a high electric field, and then collected at a low electric field²⁴. Such "field programming" permits broadening of the sample in time with minimal loss in resolving power. □

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Chromatography international

At next week's CLC '89 conference in Stockholm, Sweden, manufacturers of chromatography products from around the world will be showing their wares.

PHARMACIA LKB's model 2157 **HPLC autosampler** requires a minimum of sample and operates unattended (*Reader Service No. 101*). The injection volume of the automatic sample injector can be varied between 1 µl and 1 ml in 1-µl increments. The injection system is designed for zero dead volume, so only 3 µl of sample is necessary to inject 1 µl into the column. Mobile phase is continuously passed through the sampling unit



Pharmacia's autosampler requires minimal sample volume.

between injections to minimize carryover to less than 0.1 per cent, says the company. Sixty-nine different vials can be accommodated for continuous sampling of up to 690 injections. A cooling provision maintains sample stability, a desirable feature for amino acid analysis. The autosampler is fully programmable and can be used as a stand-alone unit or be remotely controlled by PCs and LC controllers.

The Suprex model 200A **supercritical fluid chromatography** system can now be used with photoionization detection for the analysis of organic pollutants in environmental samples (*Reader Service No. 102*). According to Suprex, combining SFC with PID offers the high sensitivity required for polynuclear aromatics, selective detection between olefinic and aliphatic compounds, and reduced inter-

ference from common sample solvents. Non-destructive detection allows use of additional detectors, and no hazardous fuels or oxidizer gases are required. Suprex says its \$36,750 (US) model 200A SFC is the only system on the market able to interface with photoionization detection techniques.

Varian is now marketing its Variable Temperature Adsorption Trap (VTAT), a **preconcentration system** for increasing the detection sensitivity of gas chromatography by trapping trace components before they enter the column (*Reader Service No. 103*). Samples are loaded into the trap when it is cold. The adsorption material used and the appropriate cold starting temperature depend upon the sample. After a known volume of sample has passed through the trap, the trap is isolated. When the chromatographer heats the trap, the sample components are driven onto the column, where normal separation and detection take place. The VTAT can be purchased with Varian's model 3000 GC for \$12,000 (US); the VTAT costs \$2,000 (US) when bought separately.

LDC Analytical says its spectroMonitor 3100X **variable wavelength detector** can make differential absorbance measurements across the UV-visible spectrum from 190-700 nm without a lamp change

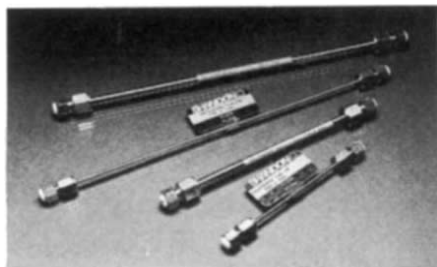


LDC's spectroMonitor scans the UV-visible spectrum.

(Reader Service No. 104). The vertical configuration, designed to conserve valuable work space, features front panel control and houses the cell, optics and electronics in a single package. The spectroMonitor's standard fluid cell yields a noise specification of $\pm 1 \times 10^{-5}$ AU at 200–300 nm and a baseline drift of less than 5×10^{-5} AU per hour, says the company. The \$5,000 (US) detector's design utilizes electrical, optical and mechanical components in a dual beam, dual path configuration to provide long-term baseline stability.

Solid phases

The Deltabond column packing material from Shandon Scientific has a **polysiloxane matrix** that enables the separation of polar molecules by SFC using pure carbon dioxide (Reader Service No. 105). Delta-



Shandon's Deltabond columns.

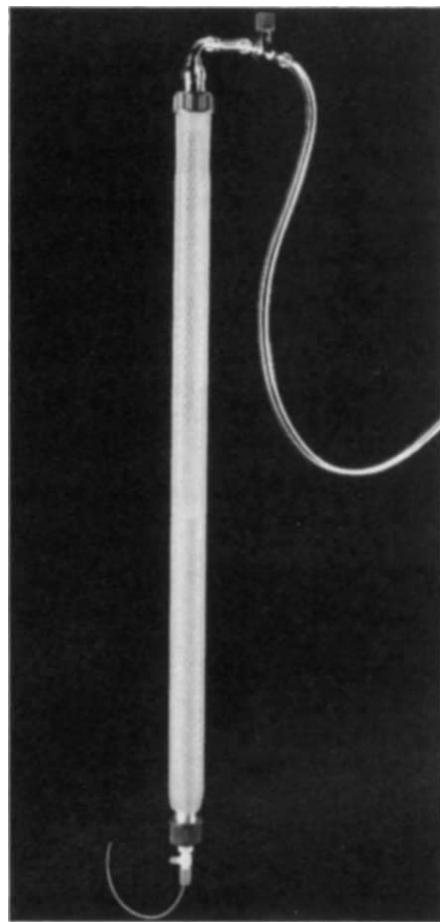
bond has a matrix of cross-linked polysiloxane groups that shield its active sites against unwanted chemical reactions, allowing it to be used without the additives required for silica packings. Deltabond columns are available with octyl, methyl, phenyl and cyano-bondings.

Eka Nobel will be exhibiting its Kromasil media and its KromaGuide computerized service for optimizing liquid chromatography separations (Reader Service No. 106). Kromasil is a high-performance **spherical silica** for liquid chromatography. The material is available in particle sizes of 5, 7, 10, 13 and 16 μm , as naked silica or derivatized with C1, C4, C8 or C18, as bulk material or in high pressure slurry-packed columns up to 2 inches in diameter. Eka Nobel says its 10 μm Kromasil typically yields plate numbers of 50 per metre.

VYDAC offers two types of silica, HS and TP, for use in **HPLC separation** (Reader Service No. 107). The spherical HS silica has a small pore size of 80 Å and a large surface area of 450 $\text{m}^2 \text{g}^{-1}$, which results in a high loading of bonded phase suitable for the separation of relatively hydrophobic compounds. The spheroidal TP silica has a pore size of 300 Å, wide enough for large biomolecules, and a surface area of 90 $\text{m}^2 \text{g}^{-1}$. Native silica columns are available packed with 5- μm HS and TP silica for \$250 (US), and with 10- μm HS and TP silica for \$209 (US).

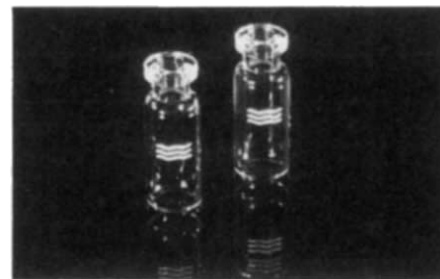
The glass menagerie

ICN Biomedicals says its new Safe-T Flash low-pressure **glass chromatography column** is safer and more convenient to use than other glass columns (Reader Service No. 108). The Safe-T Flash column features specially designed end fittings that eliminate deadspace, optimizing column efficiency for precise component separation. Grease-free PTFE/glass seals eliminate contamination problems and allow a wide range of organic solvents to be used. Screw couplings for both the columns and optional reservoirs are avail-



Safe-T Flash column from ICN Biomedicals. able to provide reliable column operation under pressure and ease connection to UV/IR detectors. A plastic mesh safety sleeve protects the analyst if the column becomes overpressurized. The column's extra-long length (75 cm) minimizes the need for an external solvent reservoir. The Safe-T Flash columns are available in sizes from 18 mm to 100 mm i.d., for \$225–350 (US).

Chromacol Gold is Chromacol Ltd's solution to unwanted chemical activity in **autosampler vials** (Reader Service No. 109). The vials are produced under stringent conditions to remove their alkaline content, which can be a problem with polar materials or low sample concentrations. Chromacol Gold is made from a specially selected inert glass that Chrom-



Chromacol's new autosampler vials.

acol says has lower levels of alkaline materials than borosilicate and soda lime glasses. Chromacol Gold vials range in price from Cdn\$308 to Cdn\$807 (Canada).

Capillary chemistry

Supelco's Petrocol 2887 capillary column is a new wide-bore **fused silica capillary column** developed to satisfy the requirements of boiling range distribution analysis (Reader Service No. 110). The column offers shorter time conditioning and quicker sample analysis than packed columns, says the company, and can potentially extend the applicable boiling range of the SIMDIS method. Supelco tests its columns to ensure proper column resolution, boiling point elution order and column bleed. The \$165 (US) Petrocol 2887 can be used with a packed column injection port and flow controller by installing a simple direct

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A new **polyimide outside coating** for capillary columns has been introduced by Chrompack (*Reader Service No. 111*). Designed to protect the naturally brittle pure fused silica, the coating has a temperature resistance that allows columns to be used up to temperatures of 400 °C isothermally and up to 430 °C in the temperature-programmed mode. The coating will not detach during repeated temperature programming, says Chrompack, ensuring strength and flexibility. The coating is now being used on Chrompack's triglyceride and simulated distillation columns.

Computer chromatography

Beckman Instruments says its System Gold for radiochromatography is the first **radiochromatograph** to combine all HPLC components as a single integrated system package (*Reader Service No. 112*). The system unites two programmable detectors: the model 166 for the detection of UV-visible absorbing compounds and the model 171 for the detection of radio-



Beckman's System Gold.

isotopes such as ^3H and ^{14}C . The model 507 autosampler is included for improved convenience and automation. An "overlay" feature allows users to compare UV and radiolabelled chromatograms on-screen, and the system's "offset" capability aligns chromatograms to account for time and volume differences between the detectors. The basic System Gold unit costs \$30,000 (US).

Perkin Elmer has expanded the data handling capabilities of its Omega-4 **chromatography workstation** to acquire data from up to four channels, with full colour graphics display (*Reader Service No. 113*). The PC-based Omega-4 System 4C combines Omega-2 software with the Epson III+ computer. The new system employs screen windows, drop-down menus and a mouse pointing device to simplify chromatography data handling. User-selected colours enable analysts to display collected data and other information, and the multitasking capability allows users to acquire data, generate a report and edit a method simultaneously. Perkin-Elmer says the \$16,500 (US) Omega-4 System 4C is compatible with all major liquid and gas chromatography instruments on the market.

ICI Instruments has a new range of **stackable HPLC modules** (*Reader Service No. 114*). The range of modules includes pumps for precise solvent delivery, a gradient computer for accurate control of composition, an advanced autosampler for automation and flexibility and detectors designed for benchtop spectrophotometry. ICI's HPLC modules may be configured together to form a stand-alone chromatography system, or be combined

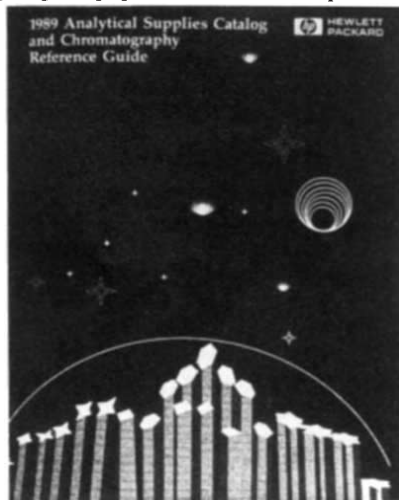


Stackable HPLC modules are new from ICI Instruments.

with instrumentation from other manufacturers. The high-pressure solvent delivery pumps can be controlled externally and deliver precise and accurate flow with virtually no pulsations, says ICI. The autosampler does not require a wash cycle because its precise metering device does not come in contact with the sample. Only 3 μl of sample are required for 1- μl injection.

The literature shelf

Hewlett-Packard's 1989 analytical supplies **catalogue and chromatography reference guide** is now available (*Reader Service No. 115*). The free 256-page catalogue contains information on products and supplies needed by most laboratories, including columns for GC and LC, syringes, paper, chemical samples and



Hewlett-Packard's free 1989 catalogue lists lots of lab essentials. supplies for GC and HPLC, UV-visible and FTIR detectors, and GC/MS and LC/MS instruments. A set of more than 100 capillary and HPLC column applications is included, as well as appendices of

chromatographic equations, miscibility and conversion tables, a glossary of chromatographic terms and a periodic table of the elements.

Philips Scientific has a new **consumables service** that guarantees same-day despatch (*Reader Service No. 116*). Through the Reflex service, which was designed to



A sampling of the products available through Philips' new consumables service.

overcome problems encountered by analytical instrument users in obtaining consumable items, a whole range of equipment, including UV cells, capillary columns, sample vials, pH meters, electrodes, buffers and hollow cathode lamps can be purchased from one source. The consumables are listed in a new catalogue that contains a special Reflex ordering card. □

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